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Choosing the top people

It is taking some time to find a successor to Sir Sam Edwards as chairman of the UK Science Research Council (SRC), the body that oversees the spending of an annual £100 million on research projects in universities, institutions and international laboratories. Sir Sam returns to the academic world in October and although feelers have been put about for several months and several people have been approached through the Department of Education and Science, it seems that no one has yet given a favourable reply.

The job is not without its attractions, because even in these days of cash limits it is still possible to pursue modest initiatives rather than simply shore up the *status quo*; indeed in some ways the power may be greater than before in such matters as postgraduate education, where the universities certainly take more note of SRC's influence and views than in more affluent times. The chairman's job could not really be said to be a post with great appeal to those who have already made their name on the scientific-political scene; strange, then, that one or two of those to whom early feelers went out were people exactly in this category. Maybe commonsense has prevailed—there are several others now being mentioned who have still a name to make; it is surely desirable that with so few scientists privy to the London political scene an imaginative choice should be added to their ranks.

The problem confronting many in this category, however, is what to do after a spell at SRC. If a professorship is resigned at the age of 45, will another professorship or university post conveniently appear at 50? If such prospects look bleak, is it possible to keep in touch with the academic world by spending a day or two every week back at the bench and giving SRC three or four days a week? If the chairman finds public service appealing, will opportunities open up for a now-experienced scientist-politician to be employed in ways that are not trivial? Would a chairman with appropriate leanings be able to find a satisfying post in industry?

That these questions have to be posed seriously and are undoubtedly running through the minds of several people interested in this appointment shows how

depressingly rigid career structures can be in Britain for senior people of high competence. The last thing that those of ability should have to do is to cling to tenured posts for fear of loss of income or serious erosion of pension; the country surely needs such people to circulate freely and to be able to put their well-experienced minds whole-heartedly to new tasks, probably administrative more than scientific, right into their sixties. There is a serious danger that by consistently failing to encourage free flow between industry, government and the academic world, Britain is wasting much of its senior intellectual resources; the appointment at SRC is just one instance of the sort of dilemma that faces those who genuinely wish to be mobile.

If a top British post in science policy is discussed discreetly by a charmed circle and names are mentioned only on promises of infinite discretion, to preserve the sensibilities of candidates, no such inhibitions seem to surround the procedure of appointing a Presidential Science Adviser in the United States. For weeks before the appointment is made names are quite openly mentioned as the pros and cons of various candidates are widely discussed. The assumption is, presumably, that aspirants to life in the public eye had better get used early on to being talked about, and that it is better to search out any rational opposition to candidates while there is still time.

Which is the better way of doing things? British concern to protect the individual or American wide exposure? Obviously the question cannot be answered in isolation from recognition of a totally different political and even cultural environment. But even so, there do seem ways in which the appointment of a few of Britain's top science-policymakers could be coupled with much more open consultation, so that the announcement does not come as a complete surprise to virtually all scientists and the name is not greeted with expressions of complete ignorance. This is a matter on which the Department of Education and Science should try to force the pace—they would find mild dissatisfaction with the present system of appointments and a general enthusiasm for more open government amongst scientists. □



European energy: where now?

Chris Sherwell looks at the immediate prospects for European energy policy with Britain in the Council chair

ASK almost anyone about energy policy in the European Community and the response, when it is not a blank stare, usually reflects deep-seated disillusion. There is no energy policy, some will imply, and there won't be one that is coherent until someone decides how much of what fuels will be needed for what purposes at which points in the future. Others say that only a policy of fully developing all possible sources of energy will fuel Europe's future growth—which, they may add, is what it is all about.

One thing is certain: the future, like nostalgia, is not what it used to be. Hence the gloom. The OECD's recent *World Energy Outlook*, for example, projecting supply and demand to 1985, pointedly urged industrialised countries to adopt more vigorous energy policies. The EEC experience certainly doesn't augur well. Francois Xavier-Ortoli, the outgoing president of the European Commission, said as much when he spoke disparagingly of the "feeble" European response to calls for a common energy policy. His successor, Roy Jenkins, stressed only last week the need to develop a coordinated policy. And the outgoing EEC Energy Commissioner, Henri Simonet, has said that EEC governments have accomplished "virtually nothing" on energy.

The theme is not always so general. Guido Brunner, the Research Commissioner who has now also taken on the energy portfolio, appealed to Britain a fortnight ago to shift position on the specific issue of the proposed Euratom loans plan; he wants a move on the sensitive issue of a minimum safeguard price for oil (msp) as well. Simonet himself has said that until the Community rids itself of "the ball and chain" of a floor price and gave a go-ahead on Euratom loans it would not be able to develop a policy for boosting alternative energy sources.

Dispelling the log-jam

Now there are hopes that the log-jam may begin to be dispelled. Mr Brunner's appeal followed a series of meetings held in various European centres by Mr Anthony Wedgwood Benn, the UK Secretary of State for Energy, who now holds the chair of the Council of Ministers because it is Britain's turn under the six-month rotation scheme which the Community operates. Mr Benn's purpose was to learn the objectives of EEC members over the period until July (when Belgium takes over), and

to discuss the ways the Council worked.

The date of the first Council meeting under Britain's chairmanship is tentatively set for 4 April. The issues it confronts extend beyond the msp and Euratom loans into the broad nuclear field and into the area of fusion and the Community's JET project as well. On all these matters progress will have been hamstrung for a year to eighteen months by the time the Council meets.

Mr Benn freely acknowledges that the Community has been unsuccessful in energy policy thus far. In an exclusive interview with *Nature* last week, he described the approach as "too scholastic", with too many people in offices with computer forecasts operating too much from the centre: officials should be made more responsive to politicians, whose shoulders must bear more responsibility.

In Europe, he says, "everything becomes a package deal" and he doesn't want a "haggling relationship". Rather, he wants a realistic one in which the needs of each country, which aren't identical, are taken seriously. The aim is to "harmonise interests", but there is no point in pillorying a country because it can't move. There should be a search for areas of common interest on which people could build a consensus.

Mr Benn says this view is shared by his European colleagues, being more open, more "discussive" and a move away from judging policy by the number of regulations or directives it produces. In energy matters decisions were not always clear and could not always be taken quickly. No policy sticks, he says recalling the Concorde experience, if it isn't also acceptable to public opinion. An energy policy which makes sense will be put together "bit by bit". And it will fall "far short of any 'federal control' of European energy; I think that is absolutely out".

It is on these lines that Mr Benn wants his Council presidency to be judged. How any success he achieves will manifest itself in the realm of decisions, though, is another matter. He is reluctant to put priorities on the solution of the many long-standing energy questions facing the Council this year: "We'll have to make progress as and when we can", he states, "and I don't want to set myself a target for a particular decision and then have every meeting which doesn't quite realise it written off as a failure".

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Mr Benn: breaking the log-jam?

Range of problems

A glance at the topics due to come up during Britain's presidency reveals the problems. They range from the barely answerable, like the rational use of energy, to the arguably unresolvable, like the protection and promotion of investment. It is the latter question which focuses upon the highly controversial issue of the msp, the background to which highlights the sort of difficulty the EEC faces.

Irrelevant symbol or careful calculation, the msp's purpose is to make investment both in the North Sea and on alternative energy sources worthwhile. Britain thinks the msp was agreed by the Community at the end of 1975 when Britain dropped her claim, as EEC's only energy exporter, to speak independently of the Community at the upcoming 'north-south' conference. And most members of the International Energy Agency (IEA), the OECD agency based in Paris, have agreed on the idea. But France—not a member of the IEA because, in her view, it encouraged confrontation with the major oil producers—has firmly resisted an msp, insisting, like Mr Simonet recently, that only an investment guaranteeing mechanism was agreed.

No one disputes that the msp issue is politically linked to the issue of the Euratom loans, a scheme under which construction of nuclear power stations might go ahead more fluidly by permitting a Community contribution. Germany and France, with their large nuclear programmes, would both benefit by it. Mr Benn himself says he was mandated twelve months ago to agree to a package in Brussels involving both these items. But there was no progress on the msp. EEC countries agreed in principle to the loans scheme, but Britain refused to sanction its implementation without agreement on the msp, even though she had no objection to it and actually stood to benefit by it. The two matters have remained inextricably linked ever since.

There is a parallel linkage in the

EEC Research Council, now chaired by Mr Gerald Kaufman from the UK Department of Industry. Its inability to agree on a site for JET, the Community's fusion project, has held up implementation of the four-year joint research programme, most recently because of French insistence that the site be at CERN if not in France itself. The two main contenders are Garching in Germany and Culham in Britain.

The possibility that, with the appointment of Mr Brunner as both Energy and Research Commissioner, these two sets of linked issues might themselves become linked is not being talked about openly. But Mr Benn, while pointing out the division of responsibilities between the two Councils, does stress that he is taking a close interest in the subject and affirms that the project is not dead. He could conceivably argue that JET is now to be seen as an energy matter rather than a research project. But for the moment he merely continues to emphasise Britain's view that the Culham site is the most suitable.

JET domino

In a sense JET is a vital domino in the efforts to stimulate some movement on European energy. Britain for one might find it more difficult to posture over certain aspects of policy if she won JET, although she is highly unlikely to relinquish her self-styled role of remaining Europe's energy equivalent of a 'bread basket' by surrendering sovereignty over North Sea oil and gas.

Yet this idea has been publicly canvassed. It was Mr Simonet's view at the end of his tenure that Britain should invite other EEC members to join in North Sea exploitation in return for the guaranteed European

market she wants even if prices fall: in other words, access to the goods for access to the market. Mr Benn is fiercely resistant to that suggestion.

He also resists the notion of dealing with one type of energy investment, in nuclear power for example, without dealing with support for other types. To him there is no trade-off to be made: if Europe is to reduce its dependence on imported energy to some 50% by 1985—its intended (if receding) goal—all energy investment must be stimulated.

But even while the haggling goes on over the msp and Euratom loans, the fact remains that Europe will still be the world's largest energy importer in 1985. That, indeed, is why some people say that Europe's nuclear policy, of which the Euratom loans are just one facet, is the most crucial aspect of its energy policy and one which simultaneously offers the best prospect of European cooperation.

The Commission is concerned that the projected nuclear future is now a mere shadow of what it was: the optimistic forecasts of 200 GW of nuclear power by the mid-1980s were reduced to 160 GW some time ago, and according to Simonet the Community would now be lucky to reach 90 GW by then. The problems here, however, stretch beyond mere construction of nuclear power stations.

The EEC countries, all members of Euratom, have so far failed to resolve the now long-standing problem posed by France, which has not signed the Non-Proliferation Treaty, regarding the arrangement for IAEA inspectors to check nuclear installations. France fears IAEA safeguards will be imposed without her consent; her Euratom

partners fear she will escape the safeguards they face. One by-product: without agreement on safeguards, a threat to future supplies of enriched uranium from abroad—at a time when at least one of the two European enrichment consortia, Urenco, is facing increasing difficulty.

The issue of reprocessing is still to come on the European front, although reassessment of the matter by the new Carter Administration could have implications for any European intention to reprocess spent fuel on an international basis. Mr Benn hopes to go to the United States to discuss such matters while Council president, although as a UK minister. But the subject of nuclear fuel supply and the nuclear fuel cycle is only slated to come up in the Council in the second half of 1977.

The work programme for Britain's half of the year includes the subjects of oil refining policy, security of oil stocks, the stockpiling of coal and the use of it in power stations. The Council under Mr Benn may not manage to embrace all these matters as well as make progress on the immediate decisions in the two meetings provisionally planned for April and June.

Past experience of the Council certainly does not offer a happy prognosis. But perhaps the man temporarily at the top can remedy that and confound his critics at the same time. It would indeed be ironic if it was Mr Benn who finally did most to promote European cooperation. That is what is now needed. Matters have reached the stage when the degree to which the old Europeans take Britain seriously over energy policy, and *vice versa*, is now to be determined. □

USA

An unprecedented debate

Colin Norman reports from Washington on the continuing discussions about genetic manipulation experiments in the United States

AFTER seven months of intense and at times acrimonious debate, the City Council of Cambridge, Massachusetts, last week lifted a moratorium it had placed on some uses of the controversial recombinant DNA genetic engineering technique at Harvard and Massachusetts Institute of Technology. Acting on the advice of a citizens' review board, the council has decided to allow the research to go ahead in Cambridge under strict safety controls and under the watchful eye of a committee of the city's residents.

While the debate in Cambridge has been coming to a resolution, however, it has been picking up momentum in many other parts of the United States. A debate without precedent, it is leading in many cases to an unusual degree of citizens' involvement in the conduct of academic and industrial research, and in the view of many could have a profound influence on future relationships between universities and their surrounding communities.

The following are some of the more important developments which have occurred over the past few weeks.

● **New York.** Legislation to control recombinant DNA research throughout New York state is expected to be introduced into the state legislature this week. If passed unchanged, it could

result in the most stringent controls so far adopted anywhere in the United States.

The legislation is a direct result of a public hearing called last October by the State Attorney General's Office, and the bill was drafted by Deborah Feinberg, an aide in the Attorney General's environmental health bureau. The legislation would essentially require that all academic and industrial research laboratories planning to conduct recombinant DNA experiments must apply for a licence from the state Department of Public Health. The Commissioner of Public Health would be empowered to draft state-wide regulations, based on the NIH guidelines but with additional enforcement provisions. Health monitoring of laboratory personnel would be required, for example, and applicants for licences would have to describe their proposed

monitoring procedures. The health department would also be given power to inspect all facilities conducting recombinant DNA experiments, to ensure that the regulations are being followed. Violations could result in a fine and suspension of the licence.

In a 60-page report accompanying the bill, Feinberg recommends that most recombinant DNA experiments should be conducted in moderate containment laboratories (P3 laboratories under the terms of the NIH guidelines), at least until the potential hazards have been better evaluated. "If it then seems safe and appropriate", she suggested last week, "they could be lowered to P2 or P1". The report is advisory, however; the bill requires the health department to set containment levels for classes of experiments.

● **California.** Two committees of the California state assembly, the health committee and the committee on energy, environment and natural resources, have been holding public hearings on recombinant DNA at which a number of prominent opponents and proponents of the research ("the recombinant DNA travelling road show", one wit has described them) testified. A bill, drafted by Marc Lappé, an official in the state health department, is under consideration by the committees, but it has not yet been introduced into the legislature.

Lappé said last week that he expects the legislature to approve legislation later this year which would at least make the NIH guidelines applicable to all facilities conducting recombinant DNA research in California. Like the New York legislation, the California bill is also expected to include provisions for health monitoring of laboratory workers and enforcement by the state government. In addition, Lappé said that the bill now under discussion would require principal investigators to obtain informed consent from other laboratory personnel taking part in recombinant DNA research.

● **Cambridge.** The widely-reported debate over recombinant DNA research in Cambridge is largely responsible for sparking the interest of other city and local governments in the issue. The city council last July imposed a three-month moratorium (later extended to seven months) on P3- and P4-level experiments at Harvard and MIT, and established a committee of Cambridge citizens to recommend the conditions, if any, under which such research should be permitted in the city. Last month, the committee suggested the P3-level work should be allowed to go ahead in accordance with the NIH guidelines, but with some added precautions. All P3 experiments should be conducted on crippled microorganisms, for example, there should be health

monitoring of laboratory workers, and a committee of Cambridge residents should be established to oversee the work, to ensure that the regulations are followed.

Last week, the council rejected, by a vote of 6 to 3, a resolution proposed by Mayor Alfred E. Vellucci to prohibit all P3- and P4-level experiments in Cambridge, and it then voted to implement the committee's recommendations. The vote, which essentially incorporated the recommendations into city ordinances, was widely regarded as an acid test of public acceptability of recombinant DNA research.

● **Princeton.** Events at Princeton University provide a unique, and in some respects, exemplary record of cautious debate and discussion before proceeding with recombinant DNA research. Nobody at Princeton was involved in developing recombinant DNA techniques, and thus no experiments were under way there before the national debate began. Last year it was decided no recombinant DNA experiments should be permitted at Princeton until the matter had been fully discussed.

Last September, the university established a special biohazards committee, under the chairmanship of ecologist Robert May, to recommend a policy for the university. The committee, which consisted of three biologists and seven people from other disciplines, unanimously approved a report last December which essentially states that the research should be permitted at Princeton, subject to controls more strict than the NIH guidelines. It suggested, for example, that no P4-level work be permitted, that the NIH guidelines be used as a minimum set of requirements for containing modified microorganisms, and that any P3 laboratories built at Princeton should meet more stringent standards.

The report then went to the University Research Board, which endorsed it unanimously and passed it on to the university's President. He took it to the Council of the Princeton University Community, a group consisting of undergraduates, graduate students, faculty and alumni, which also endorsed it unanimously. The President, William G. Bowen, intended to take the report to the university's trustees, the top governing body, at the end of January, but in the meantime, the local community was brought in. A public meeting organised by officials from the university and from the local township and borough was held to discuss the report, as a result of which a citizens' committee has been established to review the proposals. Bowen is expected to await the findings of that committee before taking the report to the trustees.

● **San Diego.** After the debate erupted in Cambridge, Mayor Pete Wilson of San Diego invited officials of the University of California at San Diego to meet with him to discuss their plans for conducting recombinant DNA experiments. They informed him of their intent to construct a P3-level laboratory in the new medical school, and invited an aide to the Mayor, Mike Madigan, to sit on the university biohazards committee. Subsequently, the city also established a citizens' committee to review the matter. The committee conducted about six months of hearings, taking testimony from a variety of people from around the United States, and it issued a report last month which is expected to be debated by the city council in the next couple of weeks.

The committee intentionally steered clear of recommending whether or not the work should be allowed to proceed at the university, since the city has little formal jurisdiction over the university. It recommended, however, that more citizens be brought on to the biohazards committee, urged the university to disseminate information on its plans for recombinant DNA research, and suggested that health monitoring of laboratory workers be undertaken.

● **Ann Arbor.** An intense internal debate took place at the University of Michigan last year over plans to conduct recombinant DNA research there. The university planned to construct P3-level facilities for recombinant DNA research, and it established a committee to review the biological hazards and a second committee, consisting of non-biologists, to look into the social and philosophical implications.

After an extended debate, the committee of non-biologists recommended, with one dissent, that the university should go ahead with its plans, and the Board of Regents, the university's governing body, subsequently endorsed the recommendation by a vote of 6 to 1.

● **Bloomington.** A public hearing was held last year by an environmental committee appointed by the Mayor of Bloomington, Indiana, to review plans by the University of Indiana to construct a P3 laboratory for recombinant DNA research. The council has so far taken no action, however, and an aide to the Mayor said last week that city officials plan to keep an eye on events, but no city-level regulation is anticipated at present.

● **The federal government.** Much of the concern which has been expressed

An oversight meant an error in "Carter's energy problem" (3 February, page 398-399): \$31,400 million and \$42,000 million should have read \$314,000 million and £420,000 million, and 100 million and 300 million should have read 1,000 million and 3,000 million. Our apologies.

in state and local debates on recombinant DNA research stems from the fact that the NIH guidelines do not apply to industry, and are not backed by any enforcement mechanism. In that regard, a little-known committee, established last year by President Ford, could prove influential. The committee, which consists of representatives of a variety of federal agencies, is looking into ways to extend the NIH guidelines to cover all recombinant DNA research in the United States. The committee, which meets in closed sessions, is expected to issue recommendations in mid-March.

According to one participant, a broad consensus has been established in the committee that the NIH guidelines should be transformed into enforceable regulations, but it seems that no existing agency has the power to enforce them. For obvious reasons, NIH doesn't want to be placed in the position of both supporting and regulating the research, and the committee is now debating whether to recommend that several regulatory agencies be given the responsibility jointly, or whether to recommend that

Congress should pass legislation to give the authority to a single agency. The basis of the regulations, in any case, will be the present NIH guidelines.

In the meantime, a bill was introduced into both the House and the Senate last week to establish a federal licensing scheme for recombinant DNA research, which would apply to both industrial and academic research. Drafted by Senator Dale Bumpers of Arkansas, the legislation is designed to enforce the NIH guidelines as a bare minimum. When he introduced the bill, Bumpers said that if he had his choice, "there would be a moratorium declared right now on all recombinant DNA research". Senator Edward Kennedy's health subcommittee is expected to hold hearings on the bill early in April, after the inter-agency committee has issued its proposals.

Underlying a good deal of the concern is a deep suspicion of what industry is intending to do with recombinant DNA technology, and those suspicions have surfaced dramatically in the past couple of weeks following the publication of a proposal by the Department of Commerce to expedite

patent applications involving recombinant DNA techniques.

The proposal, drafted by Assistant Commerce Secretary Betsy Anker-Johnson, would require patent applicants to state that they will abide by the NIH guidelines, though they would be permitted to "include an explanation of any deviations (from the guidelines) considered essential to avoid disclosure of proprietary information". That exemption has attracted criticism because it would allow industrial companies to proceed with their research without notifying anybody.

Such criticism last week prompted Joseph Califano, the new Secretary of Health, Education and Welfare, to ask Secretary of Commerce Juanita Kreps to withdraw the proposal. Dr Anker-Johnson pointed out in a telephone interview last week, however, that the proposal in fact forces industry to disclose more information than it is required to under existing regulations. Further disclosure could result in loss of patent rights in many countries which preclude the award of patents on processes for which there has been any prior disclosure. □

CANADA

Guidelines recommended

Canadians are now discussing guidelines for recombinant DNA experiments. David Spurgeon reports

AN ad hoc committee of Canada's Medical Research Council has presented its recommendations on guidelines for handling recombinant DNA molecules. The guidelines are similar to those adopted by Britain and the United States, although they are designated differently, but they go further in that they include guidelines also for experiments involving animal viruses and cells.

The committee's report was made public recently in a press conference held by the MRC, which is the federal granting agency for medical research in the universities. If adopted, the guidelines will apply to all MRC grantees, but not to industry. A federal interagency committee has been studying the question as it applies to industry. Dr G. Malcolm Brown, the MRC president, said the ad hoc committee's recommendations would be discussed at a meeting of the council near the end of February, when they could be approved, rejected or modified. It is considered almost certain, however, that the council will accept them.

The recommendations were drawn

up by a group that included researchers in the appropriate fields and also a member of Queen's University's law faculty. The committee also included a member of the Defence Research Establishment, a member of the University of Alberta's pediatrics department and an observer from the National Research Council.

The Chairman of the committee was Dr Louis Siminovitch, chairman of the department of medical genetics at the University of Toronto and geneticist-in-chief at the Hospital for Sick Children. Dr Siminovitch was influential in the MRC decision to include animal viruses and cells in the committee's terms of reference.

At present, no research involving recombinant DNA is being conducted in Canada, but some experiments have been proposed. A total of one-sixth of MRC's grants could be affected by the proposed guidelines, totalling about \$8-10 million a year, because of their effect on animal viruses and cells.

The earliest the recommendations could be put into effect would be April 1, according to Dr Brown, but Dr Siminovitch said many precautions have already been introduced. If the guidelines are approved, the MRC will insist that all recommendations for laboratory safety are carried out before granting funds. The mechanism for

enforcing guidelines is thus similar to that adopted in the United States, though different from that in Britain.

The guidelines themselves provide for six levels of containment, ranging from a statement of good microbiological laboratory practice (level A), plus suitable containment measures for agents of no known or potential risk, to requirements for very hazardous materials, such as the virus that causes lassa fever (level F). The technique and equipment described cover all aspects of laboratory research, including the design of laboratories, treatment of effluents, protective clothing and handling of animals.

In the section on recombinant DNA molecules, biological containment methods involving the use of strains of viruses and bacteria that have been altered so that they will not survive outside the laboratory, are included. In the section on cells and viruses, viruses known to cause infectious diseases, and those known or thought to be associated with cancer, are classified according to risk on the basis of their infectivity for man, and also for animals, the severity of the disease they cause, and the range of animals they can infect. Various factors, such as the type of cell, culture conditions, and the manipulations involved, are used to assign the various experiments with animal cells in culture to the containment levels described. Three levels of responsibility are described: the prin-

principal researcher, the institution where the work is performed, and the MRC. The chief responsibility lies with the principal investigator.

In its report, the ad hoc committee attempted to relate its proposed standards to those of the US National Institutes of Health and the UK Working Party on the Practice of Genetic Manipulation. "Level A requires no special design features beyond those suitable for a well-designed laboratory," the report said. The level is similar to the NIH's P1, but less stringent than the UK's Category I.

Level B requires a ventilated cabinet and is similar to the NIH P2 and the UK Category I. Level C requires a segregated room or cubicle from which all air is exhausted directly to the outside or is recirculated after passage through a high efficiency particulate air filter. In addition, all air exhausted from protective hoods must be passed through a HEPA filter. This level is more stringent than the NIH P2, less stringent than NIH P3, and similar to UK Category II.

Level D requires a directed airflow through the entire laboratory and

thence directly to the outside. All exhaust air must be filtered through a HEPA filter. This level is similar to NIH P3 but less stringent than UK Category III. Level E requires the addition of an air lock, a Type III biological safety cabinet, an emergency plan and alarm system, and a safety cabinet outside the laboratory area. It is more stringent than NIH P3 and similar to UK Category III.

Finally, Level F calls for an isolation unit geographically separate from other areas. This separation may be by means of a separate building or by a separate air system in a multipurpose building. It is the equivalent of NIH P4 and UK Category IV.

A number of experiments with uncharacterised or partially characterised DNA are forbidden at present:

- The deliberate creation of recombinant DNA expected to make harmful products, such as plant pathogens of increased virulence.
- The deliberate release into the national environment of an organism containing recombinant DNA.
- The use of recombinant DNA to

transfer drug resistance to micro-organisms not known to acquire it naturally, if such transfer could compromise the effective use of the drug in human or veterinary medicine or agriculture.

Other experiments are to be individually assessed by the MRC to determine whether they should be allowed to proceed and, if so, what containments are necessary. These include etiological agents that may pose a special hazard because of particular reasons (that is, that they do not occur in Canada); plants, animals or insects that are either poisonous or whose entry into Canada requires a special permit because of pathogenicity; and all viruses that require containment levels above B.

The report pointed out that guidelines would require periodic review in the light of new information, and that there would still be experiments for which guidelines do not provide explicit containment requirements. For these reasons, the committee proposed that the council set up a standing committee to review the needs and advise the council. □

BRITAIN

Windscale: no escape

No development connected with the Windscale plant operated by British Nuclear Fuels Ltd (BNFL) now escapes national headlines. Chris Sherwell outlines some of the latest problems

WINDSCALE remains in the front line of an increasingly controversial debate over nuclear power in Britain. What encouragement there was last week for BNFL came in the House of Commons, when at the end of a second reading debate on the Nuclear Industry (Finance) Bill a vote was recorded of 196 to 22 in favour. Among other things, the bill provides for an increase from £75 million to £300 million (£500 million by order of parliament) in the limits of the capital which BNFL can raise with government backing.

BNFL is reluctant to place any interpretation on the vote for the bill or on its content, but late last year, when expansion at Windscale came under heavy scrutiny, Mr Con Allday, BNFL's managing director, did draw encouragement from the support which he said the existing guarantees represented.

In the House last week, Mr Anthony Wedgwood Benn, the Secretary of State for Energy, implied that the bill was at least a case of prudent prepara-

tion. Although he was at pains to stress that the authority provided by the bill would not become operative without planning authority for the expansion, he said it was important that the company should be able to keep open with its customers the option of undertaking reprocessing business should that authority be forthcoming.

On both these aspects there have been some further developments recently. It was just before Christmas that Mr Peter Shore, the Secretary of State for the Environment, announced that BNFL's application to build its proposed oxide fuel reprocessing plant would require the sanction of a public inquiry. Mr Shore also indicated that such a procedure would be unlikely in respect of applications for Magnox fuel facilities and for proposed research projects.

BNFL has since submitted a separate application for the latter expansion to Cumbria County Council, but by last week had yet to announce that it proposed to go ahead with the oxide plant application.

On the related matter of the need for BNFL to keep its options open with its customers, the possibility has now emerged that, when it makes its application, BNFL will split it in two, one asking for permission to build the reprocessing plant itself, and the other

seeking permission to construct ponds in which oxide fuel might be stored pending reprocessing. Whether the latter proposal could be sanctioned without an inquiry is not clear, since no one is actually admitting that it is under active consideration. But in the House Mr Benn did manage to give a hint on the matter.

Responding to a question on the building of storage ponds, he said that it was necessary for the company to take discussions to the point of concluding provisional contracts, and that he was having discussions with BNFL "about procedures along these lines". It is thought that this might be a way for BNFL to give additional assurances to the Japanese, at the moment the most important overseas customers. A high level delegation from Japan was in Britain last week for talks with the Departments of Energy and the Environment, with BNFL and with the Cumbria County Council.

Whether the whole issue remains one for the relevant British and Japanese authorities alone, however, is another question. The United States, as supplier of nuclear fuel for stations in Japan and other countries, is in a position to exercise a veto over what happens to spent fuel earmarked for reprocessing.

BNFL are not prepared to comment on this authorisation procedure, which derives from the requirement that any transfer of fissile material of US origin

between two countries outside the USA needs the approval of the US Energy Research and Development Administration. US policy thus far has been to authorise the transfer of spent fuel for reprocessing, but the UK Department of Energy says it "may well be" that as part of the US review of non-proliferation policy "further consideration will be given to the conditions under which authorisation is given". According to the *Times*, negotiations between British and the USA on the matter have made little progress so far.

Important as this is, though, the most immediate of Windscale's problems last week appeared to be the continuing stalemate it faced in an industrial dispute which began on 25 January and which coincided awkwardly with the Japanese delegation's visit. Changing room attendants went on strike over a claim for increased allowances for working in an active area. Other workers were laid off as a result, and the strike spread, eventually affecting some 3,000 workers. After 11 days, during which reactors had been shut down and special safety procedures adopted, workers rejected a peace formula; last week no further efforts were being planned to solve the dispute, which was costing BNFL some £14,000 a day in lost revenue.

Meanwhile, worries over the health of workers at Windscale have revived. The deaths of two Windscale workers apparently suffering from cancer-related diseases have been the subject of separate inquests; open verdicts were recorded. Difficulties over secrecy being faced by BNFL lawyers handling two other cases, involving families of Windscale workers who died of cancer-related diseases, are thought to be partly responsible for last week's publication by the National Radiological Protection Board (NRPB) of a paper by Dr G. W. Dolphin, its Assistant Director (Research and Development).

The long-promised paper is substantially the one produced in September 1975 comparing the numbers of observed and expected deaths at Windscale from various cancers. It concluded that the difference was insignificant, but was much criticised, notably by the Flowers Commission on nuclear power and the environment, particularly in being limited only to those actually employed at Windscale.

With the aid of an independent consultant epidemiologist, Dr Peter Smith, BNFL is now to conduct its own two-year study of all 20,000 workers who have been employed at Windscale for any period of time in the past. Its findings will be lodged in a registry of radiation workers which the NRPB has established to produce a long-term study of those exposed to radiation. □

USSR

The web of 'secrecy'

Vera Rich reports on the case of Dr Naum Salanskii, now facing problems in seeking to emigrate from the USSR

THE prospect that this year's Diamond Jubilee of the October Revolution might be marked by a spirit of amnesty and a relaxation of pressure on dissidents and minority groups is beginning to look less realistic. A vicious attack on Andrei Sakharov in the embassy 'hand-out' publication *Soviet News*, the arrest of members of the 'Helsinki monitoring group' (including physicist Yurii Orlov and science-fiction writer Mykola Rudenko), and now the peculiar case of Dr Naum Salanskii in Vil'nyus, all indicate an intensification rather than a relaxation of pressure.

Dr Salanskii, formerly a laboratory head at the Institute of Physics of the Siberian Branch of the Academy of Sciences of the USSR, applied in 1975 to emigrate to Israel and was, according to standard Soviet practice, dismissed from his post. Before leaving the institute, however, he managed to obtain a document signed by the Director of the Institute, Professor I. A. Terskov, which stated unequivocally that "all the works by N. Salanskii were published at home and abroad in journals and other *non-secret* publications".

Dr Salanskii was nevertheless refused permission to emigrate, and on the grounds of his "secret work". With Professor Terskov now retired, Dr Salanskii found it impossible to get the new Director, Professor Pershov, to confirm his predecessor's assessment. While awaiting permission, Salanskii, like so many refusniks, became associated with Mark Azbel's illicit seminars. At the end of December 1976, when the authorities broke up the attempt of the seminar group to hold a three-day symposium on Jewish life in the USSR, several members were held for a time by the police. In Salanskii's case the matter is being taken further.

According to official statements, Salanskii is being investigated on the charge of having "slandered Soviet policy regarding the Jewish minority". The issue of secrecy also occurred several times during interrogations. On one occasion an investigator, Baku-chornis, told him that he had written to Salanskii's old institute enquiring about the alleged secrecy of his work and had already received a reply. On another occasion he was told by an official of the Central Committee of

the Lithuanian Communist Party that no such enquiry was ever made nor had a reply ever been received since 'criminal proceedings' had been started. "This seems rather strange", Salanskii commented, in a telephone conversation last week; "first they said that enquiries had been made and a reply received, and suddenly all this has disappeared".

Salanskii is still trying to discover details of the accusation, and has asked the investigator to explain the policy of the USSR towards the Jewish minority which he is alleged to have slandered. According to Salanskii, the investigator said he was not an expert in this field and must look it up in a book. "It seemed very odd to me," says Salanskii, "that he has been working on my case for seven weeks, but still is unable to explain the accusation. As I do not know the essence of the case, I am in the meantime writing the history of my application for emigration [by way of a defence]. If I am taken to court, I will be handed the accusation just before the proceedings begin, so that I cannot prepare a defence."

Salanskii's case is noteworthy for several reasons. Isolated as he is in Vil'nyus, he does not have the moral support which one finds, for example, among the Moscow group of refusniks. And the issue of 'secrecy' has once again raised its bureaucratic head, but in this case it is not only the nature of Dr Salanskii's former work which is considered a secret, but apparently the charges against him too. An early decision has been promised as to whether the charges will be taken to court, dropped, or retained for further investigation—provided, of course, that the outcome of that decision is not itself considered an official secret. □



Naum Salanskii

Threat to Polish physicist

The Workers' Defence Committee, set up last September, includes a num-

Science adviser tipped

More than three weeks after President Carter's inauguration, vacancies still abound in the federal science agencies, and the most important post, the president's science adviser and head of the Office of Science and Technology Policy, was the only top White House job unfilled by the end of last week. There have, however, been some recent signs of movement. Several candidates were said to be under serious consideration for the science adviser's

Well regarded in science policy circles, Press was a member of the president's science advisory committee and a delegate to the test ban talks in the early 1960s. He is now a member of the national science board and chairman on the National Academy of Sciences' Committee on Scholarly Communication with the People's Republic of China. Though White House sources cautioned last Friday that Press's appointment was not cut and dried, an indication of high level interest is the fact that Press met with Carter for about half-an-hour last week.

Minister's vaccine statement

Ten days after an announcement that the UK Health Service Commissioner

What was the fuss about? The Pharmaceutical Society journal article focuses strongly on the implications of a particular section of the advertisement. Three words, in fact, saying that the MRE can supply the products advertised "to any destination". This does look worrying when the products include "toxic proteins" and "animal viruses", and when the MRE

Mr Gilbert did have some comforting words in the Commons, however.

Hard selling bugs



BACKGROUNDER

Mr Gilbert also assured the Commons that the only bacterial species mentioned which could be conceivably harmful to humans were sup-

What makes the latest development newsworthy is, as Mr Gilbert implied, the *Nature* advertisement. The Ministry of Defence decided last year that it will no longer meet the cost of providing facilities and staff at the MRE for the non-military work it does (military-related work is being transferred to the nearby Chemical Defence Establishment). That means researchers in microbiology in Britain stand to see the only facility of its type go to seed unless it can stand on its feet outside the Ministry of Defence. So to drum up business, the MRE has started advertising.

is to take up the cases of four children said to have suffered brain damage after whooping cough vaccinations, the UK Social Services Secretary, Mr David Ennals, last week told the House of Commons that despite their case he could not consider claims for vaccine-damaged children in isolation ahead of a Royal Commission report on liability and compensation for personal injury.

He expressed concern at the 60% fall in whooping cough vaccinations over the past three years, and added that the government's adviser on immunisation thought the policy of offering whooping cough vaccine should not be changed as the benefits far outweighed the risks.

UK coal report

With the help of continuing high inflation and a disappointing productivity performance the costs asso-

ciated with expanding coal production in Britain to 135 million tons a year by 1985 have more than doubled from £1,400 million three years ago to £3,150 million. However, the British coal industry's tripartite group, embracing the government, the National Coal Board and the unions, shows no signs of changing the emphasis accorded to coal in the country's energy strategy. In its third report (*Coal for the Future*, free from the Department of Energy) updating progress on the ambitious 1974 "Plan for Coal", the group stresses the importance of research and emphasises the role for UK coal in EEC energy policy.

Energy unit set up

The UK Science Research Council has set up a support unit at its Rutherford laboratory to assist the energy research it funds at universities and poly-

technics. Known as the Energy Research Support Unit (ERSU), it will also conduct feasibility studies for proposed projects and undertake necessary development work for industry to adopt research programmes.

Orkney ore demo

On the day that Orkney Island council's planning and development committee agreed to recommend the rejection of an application from the South of Scotland Electricity Board to survey for uranium, the island experienced what is believed to be its first-ever demonstration when some 400 people marched in opposition to the application. The SSEB has an agreement with some land owners and may appeal to the Secretary of State for Scotland to overrule the objections. The survey is partly financed by the EEC.

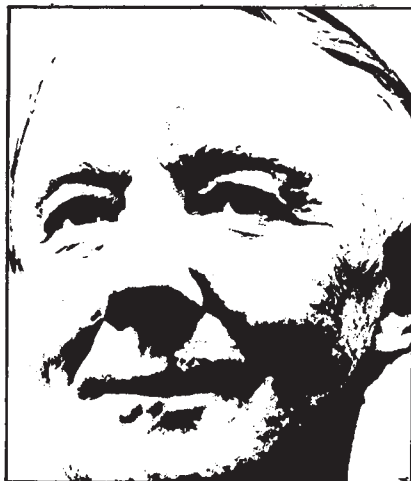
THE British landscape of small fields and hedgerows was the product of nineteenth century agriculture. Until recently, our most efficient farms were the most beautiful and provided plenty of cover for a rich selection of our native fauna and flora. British farmers consider that they are the real conservers of the countryside. This view has had official support, for most of the activities of farmers—rooting out hedges, ploughing ancient grass land, the erection or removal of all but the largest buildings—are free from planning controls. Today the whole question of rural land management and conservation needs to be re-examined.

The word 'conservation' has, unfortunately, many meanings. It can refer to the maintenance of the fertility of the soil, to preserving grass or forage as hay or silage, as well as to preserving wild animals and plants. Farmers in Britain are indeed conserving their soil and are not, as some critics suggest, destroying its structure by methods aimed at obtaining high yields today. In fact it is our most heavily cropped areas which are the most fertile; the field which gives the highest yield this year will probably give the highest next year also. Good farming is the best method of building up and maintaining the fertility of the soil.

Unfortunately, however, some recent agricultural developments, though doing wonders for food production, no longer ensure that rural Britain retains much of its beauty and diversity. In the second annual report of the Nature Conservancy Council the dangers of modern agriculture to wildlife are clearly described without

any of the exaggerated overstatements which so often detract from the jeremiads of some conservationists. Large fields of clean, weed-free cereals and productive grass leys containing only one or two cultivars are of little value to wildlife. Though the willingness of many farmers to make some compromise by retaining cover on un-

Close to the hedge



KENNETH MELLANBY

productive corners of their land is welcomed, it is clear that positive action by conservationists of all kinds is needed, and that much more money will have to be spent saving marshes and bogs, old grass and other habitats which have no useful place on the modern farm.

The situation is more serious than some of us, including scientists involved in these problems, may realise. Perfectly sound ecological research

has suggested that some changes, particularly the removal of hedges, have done less damage to wildlife than is generally imagined. Many birds, for instance, have proved to be remarkably adaptable, and can still exist in substantial numbers when the landscape has been greatly impoverished. The somewhat theoretical 'index of diversity' may not be substantially reduced when many hedges and hedgerow trees are removed. These findings must not be allowed to make people over-optimistic. The fact is that many of the most influential conservationists are ornithologists and as long as birds are not too greatly affected, they have been apt to accept changes affecting plants and other animals. Now birds are useful as delicate indicators of pollution, but the purpose of the rest of the countryside is not only to provide them with living quarters.

Birds may tolerate the removal of many hedges and trees, but the shrubs and trees lost are themselves an important part of the native flora we wish to conserve. An oak tree is not only a roosting place for pigeons and kestrels, and the source of food for some 300 different species of insect; it is in its own right the largest chunk of wildlife to be seen on many acres of farmland. Conservation must devote more attention to preserving the common and everyday features of the countryside and must make the enjoyment of these easily available to our people in general if more public support and funds are to be used to protect the countryside. The conservation of rarities and the support for esoteric ecological studies is not enough.

news and views

Ferromanganese deposits: fast, fast, slow

from Peter J. Smith

FERROMANGANESE deposits on the ocean floor were discovered by the *Challenger* expedition more than 100 years ago and have been objects of scientific study and speculation ever since. In recent years, however, their existence (especially in the form of nodules) has become well known far beyond the scientific arena, largely because of their potential economic importance and the problems this has produced in devising a fair and workable international law governing the exploitation of the seabed. Some oceanographers have expressed resentment that this familiarity has given ferromanganese deposits a popular prestige totally unjustified by their volume in comparison with the volume of all oceanic sediments—a view that would perhaps carry more weight if the renewed interest in oceanic ferromanganese and associated metals had not drawn attention to the importance of hydrothermal processes at oceanic ridges. But nodules, slabs, grains, coatings and intrusions of oceanic ferromanganese remain a fascinating scientific issue quite independent of their economic implications.

Part of their scientific attraction undoubtedly lies in their ability to grow remarkably rapidly under suitable circumstances. On page 596 of this issue of *Nature*, for example, Burnett and Piper report the discovery of a ferromanganese crust which apparently grew at a rate in excess of 800 mm per million years. This figure may be put in perspective by the claim by Scott *et al.* (*Geophys. Res. Lett.* **1**, 355; 1974) that deep sea ferromanganese crusts and nodules generally grow at rates of 1–10 mm per million years. In addition, Burnett and Morgenstein (*Earth planet. Sci. Lett.* **33**, 208; 1976) report that in a forthcoming book Ku will conclude that “both the radiometric and non-radiometric techniques employed to

assess . . . rates demonstrate conclusively that ferromanganese nodules of the deep sea grew at extremely slow rates . . . on the order of millimeters per million years.”

It is not clear from this reference whether Ku's conclusion will be held to apply to all ferromanganese deposits, in which case the validity of claims for higher rates must presumably be denied, or whether it will be a more general statement applicable only to the majority of deposits. Either way, many claims for higher rates have certainly been made. One of the earliest was by Pettersson (*Medd. Oceanog. Inst. Goteborg, Ser. B* **2**, 1; 1943) who determined a growth rate of 1,000 mm per million years for a manganese nodule by measurement of its radium content. Using the same method, Von Buttlar and Houtermans (*Naturwissenschaften* **37**, 400; 1950) later found rates of 600–1,300 mm per million years for several other nodules. And more recently Scott *et al.* have reported growth rates of 130–250 mm per million years for a manganese oxide crust, and Moore and Vogt (*Earth planet. Sci. Lett.* **29**, 349; 1976) have obtained rates of 1,000–2,000 mm per million years for similar crusts.

Some of the most astonishing rates of growth, however, were discovered by Goldberg and Arrhenius (*Geochim. cosmochim. Acta* **13**, 153; 1958) who reported finds on the sea floor of an approximately 50-yr old naval shell with a Mn-Fe oxide coating almost 30 mm thick and a World War II shell with a coating about 15 mm thick, indicating growth rates of about 60,000 and 100,000 mm per million years respectively. Admittedly these deposits formed in shallow near-continental waters and may thus not be comparable to deep sea deposits. On the other hand, their ages could be inferred directly without recourse to radio-

metric and other physicochemical methods of dating, and so the determined growth rates are fairly certain, at least to the correct order of magnitude. Unfortunately, this is not so for older ocean floor deposits—a situation which leaves room for doubt about the validity of measured growth rates in general.

Indeed, Burnett and Piper acknowledge the possibility of doubt in showing that different methods of dating lead to quite different estimated growth rates. Specifically, the rate of 800 mm per million years was obtained by hydration-rind dating and by measuring an excess ^{230}Th exposure age; uranium-series dating gave a much lower rate of about 48 mm per million years. Burnett and Piper give apparently valid reasons for supposing the latter rate to be incorrect, thus making a case which Burnett and Morgenstein recently developed at greater length. In the previous study, of four Pacific ferromanganese deposits, two had uranium-series and hydration-rind dates in agreement whilst two produced significant discrepancy. In each of the two disagreeing cases the uranium-series date was the lower, further study suggesting that it was incorrect because of a thorium distribution controlled by processes in addition to radioactive decay.

In spite of such problems, however, it seems likely that when dating methods have been fully assessed and possible sources of error identified, many of the higher (as well as the lower) growth rates claimed for ferromanganese deposition will be substantiated. In other words, there is apparently a wide variation in growth rates which must be taken into account in deciding how deposits form. Over the past 100 years many models have been proposed to explain the origin of ferromanganese deposits, including

the interaction of sea water with submarine volcanic products, direct precipitation from sea water of land-derived ferromanganese, and the remobilisation of ferromanganese from oceanic sediments or underwater igneous outcrops. But although these processes may play some part in some or all circumstances (for example, direct precipitation may largely account for the high deposition rates often observed in near-continental shallow water), high rates of growth observed in samples from oceanic ridges suggest the existence of at least one other major source.

In recent years this has been identified as hydrothermal activity, which in this context means the circulation of seawater within cracks and pores of hot newly-formed basaltic crust, the leaching of metals from that crust and the consequent metal enrichment of the

resulting hydrothermal fluids. Hydrothermal activity is the most likely candidate for several reasons, not the least of which is the need to invoke it for other purposes such as to explain the distribution of heat flow in the vicinity of oceanic ridges. But more directly, by extrapolating from the East Pacific Rise, Lyle (*Geology* **4**, 733; 1976) has recently estimated the total input of hydrothermal manganese to the world's oceans to be about 9×10^8 kg yr⁻¹, or nearly four times the dissolved load of manganese carried by rivers. Unfortunately, there are no reliable figures available for the detrital input of manganese, and so the total contribution from the continents remains unknown. Nevertheless, by any standards hydrothermal activity is evidently an important source of oceanic ferromanganese. □

How does T4 turn off *E. coli*?

from Christopher G. Goff

It has been clear for a long time (on the time scale of molecular biology) that bacteriophage T4 can turn off the expression of *E. coli* genes in its host cell, while continuing to direct synthesis of its own gene products (Benzer *Biochem. biophys. Acta* **11**, 383; 1953; Hayward and Green *Proc. natn. Acad. Sci. U.S.A.* **54**, 1675; 1965). This is true despite the fact that expression of T4 genes depends throughout infection on the *E. coli* transcription enzyme and *E. coli* protein synthesis machinery. A satisfactory explanation of this "host shutoff" has been hard to come by. We know that T4 degrades *E. coli* DNA, while protecting its own genome from self destruction by replacing its cytosine with hydroxymethylcytosine, not recognised by T4 nucleases. But host gene products stop appearing well before *E. coli* DNA is broken down. The shutoff mechanism is obviously complex; it may involve more than one of the numerous T4-specified changes which occur in both the transcription and translation apparatus of *E. coli* after infection. Nevertheless two recent papers suggest how one specific T4 protein bound to *E. coli* RNA polymerase may be a major factor in the shutdown of *E. coli* RNA synthesis after infection.

There are four T4 proteins non-covalently attached to *E. coli* RNA polymerase after T4 infection (Stevens *Proc. natn. Acad. Sci. U.S.A.* **69**, 603; 1972). Two of these, the products of T4 genes 33 and 55, are positive con-

trol elements needed for synthesis of the major class of T4 RNA appearing late after infection (Horvitz *Nature new Biol.* **244**, 137; 1973; Ratner *J. molec. Biol.* **89**, 803; 1974). Another of these four proteins, 10,000 daltons in size but genetically undefined, apparently alters or inactivates the sigma subunit of RNA polymerase (Stevens & Rhoton *Biochemistry* **14**, 5074; 1975), at least *in vitro*. The fourth, 15,000 daltons in size, has until now been mysterious; no lethal mutations have defined its gene or its function. But Snyder's lab at Michigan State has recently reported (Sirotkin, Wei & Snyder *Nature* **265**, 28; 1977) that the *alc* mutants isolated last year by Snyder, Gold, and Kutter (*Proc. natn. Acad. Sci. U.S.A.* **73**, 3098; 1976) affect this T4 protein. These *alc* mutants were isolated in a multiply-mutant strain of T4 unable to synthesise hydroxymethylcytosine and unable to degrade normal cytosine-containing DNA. One might expect these phage, whose DNA contains cytosine, to be viable; but they are not, because (it turns out) they cannot make the 'late' class of T4 mRNA. A single additional mutation—in the *alc* gene—permits these cytosine-containing T4 to transcribe late RNA and grow. Snyder's lab now finds that many *alc*⁻ phage fail to attach normal amounts of the 15,000 dalton protein to *E. coli* RNA polymerase. This suggests (but doesn't yet prove) that *alc* codes for the 15,000 dalton protein. Moreover, they find

that several times more *E. coli*-specific RNA continues to be made after infection with *alc*⁻ T4 than with *alc*⁺ T4. Here again, more data are needed, but it is possible that the presence of functional *alc* protein prevents RNA polymerase from transcribing cytosine-containing DNA, whether that DNA encodes T4 genes or *E. coli* genes. This hypothesis could explain why no conditional lethal 15,000 dalton protein mutants have been found in normal T4: the protein would not be necessary for transcription of the usual hydroxymethylcytosine-containing phage DNA, and failure to shut off host RNA synthesis would not be lethal either.

How might the *alc* protein block transcription of cytosine-containing DNA? Baralle and Travers (*Molec. gen. Genet.* **147**, 291; 1976) have recently found that *E. coli* RNA polymerase purified from T4-infected cells (and containing the 15,000 dalton T4 protein in question) has a lower affinity for *E. coli* rRNA promoters than does *E. coli* RNA polymerase from uninfected cells. This is true even when both forms of the enzyme are given excess initiation-specificity subunit sigma. The difference is very likely due to the 15,000 dalton protein; the authors have evidence that Stevens' 10,000 dalton T4 protein and an ADP-ribose residue added to the α subunit of RNA polymerase (Goff *J. biol. Chem.* **249**, 6181; 1974) are not responsible. In some *in vitro* conditions which permit normal *E. coli* RNA polymerase to initiate synthesis rapidly at rRNA promoters, the enzyme from T4-infected cells is unable to use those promoters at all. In these conditions, then, the 15,000 dalton protein seems to cause essentially complete shutoff of rRNA synthesis. The big question in any such *in vitro* experiment is, of course, "What are the conditions *in vivo*?" but certainly the 15,000 dalton (putative *alc*) protein could have a role in host shutoff. Travers has suggested before that RNA polymerase can "flip-flop" between a form active on ribosomal RNA promoters and one or more forms active on mRNA promoters, and that guanosine tetraphosphate (ppGpp), associated *in vivo* with the stringent response, forces RNA polymerase out of its rRNA-specific conformation (*Molec. gen. Genet.* **147**, 225; 1976). In their *Molecular and General Genetics* paper, Baralle and Travers show that the T4 infection has the same effect on RNA polymerase as ppGpp; the nucleotide cannot further reduce the fraction of rRNA made by the enzyme from T4-infected cells, in any *in vitro* conditions. So the *alc* protein may be an allosteric effector locking RNA polymerase in a form inactive on at least some *E. coli* promoters—and con-

ceivably inactive on T4 promoters as well, if they contain cytosine rather than the usual hydroxymethylcytosine.

There are still a lot of questions here, even if Snyder and Travers are completely right. Baralle and Travers' *in vitro* experiments reveal no reduction in transcription of $\phi 80$ mRNA from cytosine-containing DNA when the 15,000 dalton protein is present. Would the *alc* protein alone prevent use of cytosine-containing T4 promoters *in vivo*? Mailhammer *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **72**, 4928; 1975) saw a significant reduction of *E. coli* β -galactosidase synthesis in a coupled *in vitro* protein synthesising system when they used RNA polymerase from T4-infected cells; they attributed the apparent shutoff of *E. coli* mRNA synthesis to the ADP-ribosylation of the α subunit. *In vitro*, and presumably *in vivo*, active sigma protein is required for transcription of both *E. coli* and T4 genes. Yet Stevens' 10,000 dalton T4 protein inhibits sigma function *in vitro*; this T4 protein has been largely ignored in the studies mentioned here. Possibly the *alc* protein, ADP-ribosylation of α , and the 10,000 dalton protein all play a part in shutoff of *E. coli* transcription when T4 infects the cell. □

Bacterial adherence

from a Correspondent

THE ability of many pathogenic as well as beneficial microorganisms to attach to a particular surface of their host is one obvious requirement for colonisation and in some cases invasion of that host. The initial attachment of bacteria to the nasal or intestinal mucosa, for example prevent them from being washed away or dislodged by a cough or a sneeze. But surprisingly, little is known about the initial interactions between colonising bacteria and the host tissues. Some recent work suggests, however, that the interactions may be considerably more complex and specific than previously realised and some of the surface molecules involved have been identified.

Since carbohydrate, in the form of glycoproteins or glycolipids, is an important constituent of most cell surfaces, including bacterial and mucosal surfaces, a role for these substances in bacterial adherence has been suspected for more than 20 years. Duguid and colleagues, following an earlier observation of Collier and de Miranda, were the first to show this directly by using mannose to inhibit binding of *Escherichia coli* to intestinal epithelial cells

(*J. Path. Bact.* **74**, 397; 1957). A large number of other simple sugars were tested and found to be inactive. The bacterial surface component responsible for adherence appeared only in old non-growing cultures about the same time as filaments appeared. These original observations have now been taken a step further by Ofek, Mirelman and Sharon (see this issue of *Nature*, page 623). They show that in agreement with the earlier results binding of non-pathogenic K12 or B strains of *E. coli* to human cells is specifically inhibited by mannose or mannosides. Binding is also shown to be inhibited by a mannose-seeking plant lectin (concanavalin A) which presumably competes with the bacterial surface component for epithelial cell-surface mannose receptors. These receptors are destroyed by mild periodate treatment of the epithelial cells, which is consistent with the presence of mannose-like receptors, since these groups are particularly susceptible to periodate oxidation. The bacterial component (a lectin) is extracted into simple buffers without detergents and is presumably part of the filaments seen in adhesion-competent organisms, rather than being an integral part of the bacterial envelope. The soluble component, as yet unpurified, agglutinates cells at high dilution and inhibits attachment of *E. coli* to cells. It is a protein or perhaps glycoprotein which is destroyed by trypsin or heat.

The nature of the periodate-sensitive epithelial cell receptors carrying mannose and recognised by the bacterial lectin is unknown. Not surprisingly, mucins present in intestinal tract secretions, which contain little mannose, were found by Duguid and Gilles to be poor inhibitors of bacterial adherence. Other glycoproteins containing more mannose and binding concanavalin A are presumably the functional receptors. Perhaps these glycoproteins are more intimately associated with the mucosal cell surface membrane since ultimately invasion of the mucosal lining (perhaps by phagocytosis) takes place. Alternatively, these later membrane-mediated events in bacterial invasion may involve additional interactions between the epithelial cell surface and other bacterial surface components.

A wide range of *E. coli* strains show mannose-sensitive binding to host cells and to erythrocytes in haemagglutination (Jones & Rutter *J. gen. Microbiol.* **84**, 135; 1974), although not all, even some which produce enterotoxin, are pathogenic. Two *E. coli* surface components appear, however, to correlate exactly with pathogenicity in pigs and calves. These are the K88 and K99 antigens respectively (Jones & Rutter

op. cit.; Burrows, Sellwood & Gibbons *J. gen. Microbiol.* **96**, 269; 1976). The K antigens are proteins, most probably glycoproteins, since a small amount of carbohydrate is present even in the purest preparations, and can be distinguished from the lectin-like substance by their binding to mucosal epithelial cells in the presence of mannose. Like the lectin, however, their production by bacterial strains always correlates with the appearance of fine branched filamentous projections covering the surface of the cell. Together with the mannose-binding lectin, the K antigens may form a part of the projections and if so are then favourably placed to interact with the surface of the preferred host cell.

So far analogous surface antigens on human enteropathogenic strains have not been identified unequivocally but it is reasonable to assume that such components, in addition to the lectin identified by Duguid and Ofek *et al.*, are necessary for the successful colonisation and invasion of the human intestinal tract as opposed to the normal colonisation of the colon.

Clearly, the relation of mannose-binding lectin and the K antigens in bacterially-induced neonatal diarrhoea is of great interest, since this disease is now one of the major causes of death in small children in developing countries (Sack *A. Rev. Microbiol.* **29**, 333; 1975) as well as being a severe economic burden in livestock production. In the future, more detailed identification of those surface components involved in bacterial adherence could lead to rational schemes for the preparation of broad range vaccines effective in blocking or reversing bacterial attachment. Such vaccines may have advantages over more conventional vaccines which aim to neutralise toxins which are active at a later stage in infection. □

Optical fibres in communications

from a Correspondent

ONE of the consequences of rapid growth in demand for telegraphic communications is a continuing search for more efficient ways of transmitting information over large distances (see *Nature* **261**, 371; 1976). High rates of signalling call for transmission lines with high frequency response; hence our telephone services now make use of microwave devices at very high fre-

quencies of the order of 10^9 Hz. It was inevitable that in the search for ever higher transmission rates electromagnetic waves in the visible range would be considered. With frequencies of the order of 10^{15} Hz, light waves are potentially capable of solving line-capacity problems for the foreseeable future.

One difficulty is that light is so easily absorbed. Line-of-sight transmission through the atmosphere is too unreliable to be of use, and research has concentrated on channelling the light inside fine glass fibres which can be made at relatively low cost and have the flexibility associated with ordinary wires (see for example, Chynoweth *Phys. Today* **29**(5), 28; 1976). If sufficiently transparent glasses can be found, these fibres will be the telephone wires of the future. At the recent Solid State Physics Conference in Manchester, UK, glasses having losses as low as 0.5 dB km^{-1} were reported in a review lecture given by W. A. Gambling.

Disordered materials in general and glasses in particular present some of the most difficult problems in solid state physics. Absorption of photons takes place by electrons which are thrust into unoccupied states of higher energy. In crystalline materials transparency results when there is a gap in energy between occupied and unoccupied states which is too wide for the photon energy to excite electrons across; any residual absorption is due to impurities or imperfections in the crystal. In a glass there is no well-defined energy gap and its transparency must be explained in another way; despite the absence of an energy gap there exists a range of energies in which the electronic levels are localised rather than extended over the whole of the material. Transparency in glass comes about because, although there may be empty states of the correct energy to receive an electron which has just been excited from a localised state by a photon, these empty states are almost certainly localised in a different part of the glass and so are not available to receive the electron. Thus a glass may have excellent transparency limited only by scattering from statistical irregularities in density frozen in at the solidification temperature, provided it contains no impurities which themselves can absorb light. Iron and copper are particularly troublesome contaminants and must be reduced to the level of 1 part in 10^8 for really low losses.

To achieve such purity novel methods of glass manufacture are used. In one method silicon tetrachloride gas is reacted with oxygen to deposit silica on the inside of a quartz tube. Dopants

can be introduced into the gas to vary the refractive index of the deposit, which is important for controlling propagation of light in the fibre. After sufficient silica has been deposited, the tube is collapsed and the fibre-drawing process begun. Several kilometres can be pulled from a single tube.

Currently it is possible to produce optical cables with low enough losses ($\sim 4 \text{ dB km}^{-1}$) to allow distances of several kilometres between repeater stations, and test lines have already been installed near Ipswich in the UK by the Post Office. Developments in glass technology already in hand should assure the economic advantage of the new method of transmission in the near future. $\square$

HPRT mutants

from Dallas Swallow and Sue Povey

MUTANT cells deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT) have been isolated from cell cultures of a variety of mammalian species. These deficient cells have been selected, in many cases after mutagen treatment, in medium containing purine analogues such as 6-thioguanine which are toxic to cells containing HPRT. However, the nature of the mutations involved has been the subject of much debate. Whereas some of the mutants can most readily be explained by mutations in the X-linked structural HPRT locus, as an altered HPRT protein has been identified, many are probably regulatory. This was originally suggested by the lack of the expected relationship between cell ploidy and mutation rate (Harris *J. Cell Physiol.* **78**, 177; 1971; Chasin *J. Cell Physiol.* **82**, 299; 1973) and further, by the occasional reappearance of apparently normal rodent HPRT following somatic cell hybridisation between a rodent cell line deficient in HPRT and a normal human cell (Watson *et al. Expl Cell Res.* **75**, 401; 1972; Shin *et al., Nature new Biol.* **241**, 194; 1973; Croce *et al. Proc. natn. Acad. Sci. U.S.A.* **70**, 2590; 1973).

In a recent paper Milman and co-workers have examined HPRT in the aneuploid human cell line HeLa, using an elegant technique characterising the enzyme in terms of isoelectric point and subunit molecular size (*Proc. natn. Acad. Sci. U.S.A.* **73**, 4589; 1976). The protein synthesised by these cells was labelled with ^{35}S -methionine and cell extracts subjected to isoelectric focusing followed by electrophoresis in sodium dodecyl sulphate. In the two-

dimensional autoradiograph obtained, the spot corresponding to the usual HPRT was identified by several criteria including its removal by immunoadsorption using specific anti-HPRT antiserum. This spot was absent in each of 24 6-thioguanine resistant colonies isolated from HeLa after mutagen treatment. One of these HPRT⁻ mutants, however, produced a new spot, different in isoelectric point but of the same subunit molecular size, and also cross reacting with the anti-HPRT serum. Five independent revertants of this 'missense' mutant, able to grow in MTH medium (containing methotrexate, thymidine and hypoxanthine) which selects for the presence of HPRT, were isolated. All of these, even after repeated cloning, contained spots corresponding to both the usual and the abnormal HPRT proteins. From these revertants further 6-thioguanine resistant colonies could be readily obtained and these had again lost the 'usual' HPRT spot.

If one accepts that the revertants were indeed true clones the simplest explanation of this intriguing experiment is that the loss of HPRT in the 'missense' mutant was due to a mutation at the structural locus, and that the revertants represent activation of some HPRT locus specifying an enzyme indistinguishable from the usual one, but not normally expressed. As HPRT is known to be multimeric (see, for example, Olsen & Milman *J. biol. Chem.* **249**, 4030, 4038; 1974) it would be of interest to examine these revertants using a technique which might demonstrate heteromeric isozymes. The existence of such hybrid forms would confirm that both genes are active in the same cell at the same time.

It is not entirely clear whether HeLa resembles other clonal cultures derived from female somatic cells in having only one active X chromosome. However, if this is the case, then an attractive hypothesis to explain the findings is the reactivation of the HPRT gene on the normally inactive X chromosome. Although many attempts to reactivate the inactive X chromosome in cultured somatic cells have not been successful (see for example Comings *Lancet* **ii**, 1137; 1966; Migeon *Nature* **239**, 87; 1972), good evidence for reactivation of the HPRT locus was obtained in one series of human-mouse hybrids (Kahan & Demars, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1510; 1975). In this experiment the authors were able to identify both X chromosomes by virtue of one's being present as an X-autosome translocation and also by heterozygosity for the X-linked enzyme, glucose-6-phosphate dehydrogenase (G6PD) as well as HPRT

deficiency. HPRT⁺ clones were selected using HAT medium (hypoxanthine, aminopterin and thymidine). All the clones contained human HPRT, as identified immunologically, and the inactive X chromosome which carried the normal HPRT gene, but not the active X chromosome. The other X-linked enzymes, G6PD and phosphoglycerate kinase, were not detectable suggesting that the derepression was very localised. If reactivation of the HPRT locus on the inactive X chromosome does account for the re-appearance of HPRT in the HeLa clones of Milman *et al.*, it would be interesting to test other X-linked loci in these cells.

It has been deduced from family studies that Henrietta Lacks from whom the HeLa line was derived was a heterozygote for G6PD (type BA), al-

though the cell line HeLa is G6PD A (Hsu *et al. Science* **191**, 392; 1976). Thus it would be interesting to test G6PD in these clones to look for the derepression of the G6PD B allele on the inactive X chromosome.

Future experiments may throw some light on the mechanism of the maintenance and possible reversal of X-inactivation. It is unclear whether a similar explanation could account for some of the rodent revertants, since the X chromosome constitution and indeed the sex of origin of the various long-term rodent lines are in many cases unknown. However, it is possible that some completely different phenomenon which might have more general application to autosomal loci, will be found to explain the reappearance of HPRT in the experiments of Milman and others. □

More Luna results

from J. A. Edgington

LAST year's successful flight of Luna 24, the third Soviet spacecraft to return lunar samples to Earth, coincided with the Viking landings on Mars and attracted less attention than it deserved. Its core sample from Mare Crisium is now being examined in Moscow and Western scientists hope to receive some of this material soon. Under a US-Soviet agreement the US has provided 3 g from each of the six Apollo flights, and the Russians have reciprocated with 3 g of Luna 16 and 2 g of Luna 20 sample, out of total sample returns of 100 g and 50 g respectively. In addition, 1 g from each Luna mission was donated to French investigators, and 500 mg to British scientists.

All but about 100 mg of each UK sample was divided between eight investigating teams, and accounts of the separation procedures, together with experimental results from some of the investigators, have recently appeared in *Phil. Trans. R. Soc. (A284, 131; 1977)*. Luna 16 landed in Mare Fecunditatis in 1970, and Luna 20 in a nearby highland area in 1972; papers by the American and French investigating consortia appeared some time ago (see *Geochim. cosmochim. Acta* and *Earth planet. Sci. Lett.* for April and September, 1973). The present articles, perhaps because of their relatively leisurely publication, provide good examples of small sample techniques and of careful data interpretation.

For example, N. N. Greenwood *et al.*

(University of Leeds) have used Mossbauer spectroscopy to estimate the relative abundances of iron-bearing minerals, and their interpretation of the spectra, based on experience with Apollo samples, is somewhat different from that of the Russian workers (for example, T. V. Malysheva, *Proc. 3rd. Lunar Sci. Conf.* 105; 1972) and generally more cautious. However, one feature consistently observed in lunar fines material, a pronounced asymmetry in the pyroxene absorption spectrum, is definitely ascribed by the Leeds group to the presence of extremely small particles of metallic iron, which moreover they have shown to be concentrated in the surface layers of the fragments. At the University of Newcastle, S. K. Runcorn's group has shown that this iron is superparamagnetic and exhibits an inverse square law spectrum of grain volumes, extending to diameters much less than 13 nm.

The origin of this free iron, which is found in both mare and highland soils, is a topic of great current interest. It is non-meteoritic, being almost nickel free, and is thought to be produced by preferential sputtering by the solar wind. If so one might anticipate the presence of implanted carbon in association with it, and G. Eglinton's group at the University of Bristol has found just this. In place of the pyrolysis method, which determines only total carbon content, the Bristol group have developed an acid dissolution technique using deuteriochloric acid,

DCI, for their lunar sample studies. Trapped hydrocarbons release mainly CH₄ and may thus be distinguished from the acid-hydrolysable carbon, mainly the free element, which is released as CD₄. Eglinton *et al.* show that the content of hydrolysable carbon correlates strongly with iron content and magnetic susceptibility, although only for the Luna 20 samples did they note an increase in hydrolysable carbon with diminishing grain size. This may reflect the fact that Luna 16's basaltic soil is derived from a material of lower melting point than the gabbroic soil returned by Luna 20. The arguments used here are complex and will certainly be examined closely, though Eglinton's group has recently presented clear evidence for the intimate association of carbon and α -Fe in synthetic materials (Jull *et al. Nature* **262**, 566; 1976).

Like the Bristol workers, S. H. U. Bowie and colleagues (Institute of Geological Sciences) have produced very precise results from exceptionally small samples. Their measurements of oxygen isotopic ratios show an enrichment of ¹⁸O (of about 0.65%), compared with terrestrial ocean water, in good agreement with previous measurements and apparently typical of the lunar regolith. This isotopic differentiation is also usually attributed to the sputtering action of the solar wind, so its value should relate to the exposure time of the material. Bowie's group find a complex variation of enrichment with grain size, and suggest that this may most reasonably be explained by the presence at one locality of impact ejecta from many distant sites, each with a different exposure history.

The last paper of the collection—by P. H. Cadogan and G. Turner (University of Sheffield)—describes the determination of ⁴⁰Ar-³⁹Ar ages of four samples, two each from Luna 16 and 20. The former yield an age of 3.4-3.5 Gyr for the Fecunditatis basalts, in excellent agreement with the Caltech group (*Earth planet. Sci. Lett.* **13**, 368 and 375; 1972). Apollo samples from the other major basins all show a range of ages of 0.2-0.3 Gyr, and the present lack of evidence for such an extended period in Fecunditatis cannot be regarded as significant on the basis of so few measurements. A metaclastic fragment from the Luna 20 sample, probably typical of the adjacent Crisium basin, yielded an age of 3.9±0.1 Gyr.

Cadogan and Turner assign a minimum age of 4.3±0.1 Gyr to their Luna 20 anorthosite fragments. There are now many highland samples dated to this period, and controversy has developed over how to interpret these ages. Are they evidence for a lunar

cataclysm, comprising a few major basin-forming events, which ended 3.9 Gyr ago? Alternatively, can they be accounted for by a steadily decreasing cratering rate, resulting in a statistically-determined distribution in which individual ages reflect the latest event to reset the Ar clock? Cadogan and Turner favour the calaclysmic theory. Samples from Luna 24 should arrive at Houston in the spring and radiometric results from them are eagerly awaited. □

Morphogenesis of the mammary gland

from John K. Heath

UNDERSTANDING the mechanisms underlying the formation of organs poses a formidable problem to the developmental biologist. It is known that in most systems interactions between the epithelium of the organ rudiment and its adjacent mesenchyme are involved, but these interactions have proved to be complex. Two recent pieces of work which use the mouse mammary gland, present new experimental approaches which may provide an insight into this problem.

If the mammary epithelium is cultured in contact with mammary mesenchyme the tissues undergo typical mammary morphogenesis and differentiation, but if the epithelium is cultured alone its development is curtailed. If salivary gland is used as the source of mesenchyme, however, the subsequent morphogenesis of the mammary epithelium is typical of the salivary epithelium (Kratochwil *Devl Biol.* **20**, 46; 1969), but the biochemical properties of the mammary epithelium in this case, however, are similar to normal mammary glands *in situ*. Sakakura *et al.* (*Science* **194**, 1439; 1976) have now taken submandibular salivary gland mesenchyme from mouse embryos 14 days after conception and combined it with mammary epithelium from 16 day post-conception female embryos. After a brief period of culture the combined tissues were transferred to a site under the kidney capsule of adult female mice. Subsequent histological examination of the transplants revealed the typical salivary pattern of morphogenesis. But if the mice carrying the grafts were mated and allowed to give birth and lactate, the ducts in the graft became distended with milk. Biochemical studies showed the transplanted tissues to be producing amounts of the B protein of lactose synthetase similar to those found in lactating mammary glands *in situ*. These findings show that although the

mesenchyme plays an important part in determining the overall form, the cytodifferentiation of the gland is either already determined by 16 days or is independent of the underlying mesenchyme.

The use of heterologous mesenchyme in recombination experiments is common. But by the time experimental recombinations can be made many interactions involved in the formation of the organ may have occurred. Another problem is that although interactions can be demonstrated it is hard to tell which tissue takes the lead in determining the subsequent path of development. These difficulties have been circumvented by the exploitation of a mutant gene affecting the differentiation of the mammary gland (Kratochwil and Schwartz *Proc. natn. Acad. Sci. U.S.A.* **73**, 4041; 1976).

The mammary gland of the male can be distinguished from that of the female by the fourteenth day of gestation since it undergoes regression and either disappears completely or remains in a rudimentary state (depending on the strain of mouse used). The mesenchyme surrounds an epithelial bud and begins to condense around it. The bud then starts to undergo necrosis and the 'stalk' of the bud is cut off. This pattern of regression can be inhibited by destruction of the foetal testes and can be induced in females by administering testosterone. The involvement of exogenous substances such as hormones would seem only to complicate an already difficult situation. However Kratochwil and Schwartz have shown how the action of testosterone can be used as a starting point for unravelling the interactions involved in the process of regression.

They asked whether the hormone exerts its effect on the mesenchyme or the epithelium or both. The key to the solution of this problem was the use of the X-linked mutant 'Testicular Feminisation' (Tfm), tissues of which are insensitive to testosterone (probably due to a malfunctioning hormone receptor molecule). Although a X^{Tfm}/Y mouse is genetically male it lacks male secondary sex characteristics, and in particular the mammary glands do not show the typical testosterone-induced regression. Kratochwil and Schwartz combined epithelium and mesenchyme from X^{Tfm}/Y and X/Y embryos and observed the effects of testosterone on their development. The regression response occurred only if the mesenchyme was wild type. This implies that testosterone initially acts on the mesenchyme and that the subsequent fate of the epithelium is determined by the mesenchyme. An interesting corollary of this observation is the implication that the necrosis of the epithelium is the result

of killing by the mesenchyme as opposed to the cell-autonomous suicide typical of cell death in other systems.

The exploitation of appropriate mutants has proved of enormous value in other developmental problems and Kratochwil's work has opened up the possibility of their use in the study of organogenesis. In addition these experiments may lead to the use of other hormone-triggered tissue interactions for looking at epithelial/mesenchymal interactions. □

Hot white dwarfs and the EUV

from M. G. Edmunds

APART from the Sun, very few astronomical sources have yet been detected in the extreme ultraviolet (EUV) wavelength region 100–1,000 Å. The flight of the Apollo-Soyuz mission in July 1975 provided a platform for a grazing-incidence mirror telescope with a filter photometer, and the detection of two sources outside the Solar System has been reported. Both sources have good positional identification with white dwarf stars, and the second of these (Margon *et al. Astrophys. J. Lett.* **210**, 79; 1976) is Feige 24, a well-known spectrophotometric standard at visible wavelengths.

The EUV continuum observations have implied a considerably higher atmospheric temperature for these white dwarfs than had previously been assumed. At optical wavelengths the continuum radiation energy distribution is fairly insensitive to the atmosphere parameters, but model atmosphere calculations suggest that the fluxes in the EUV are more sensitive, especially shortward of the once-ionised helium absorption edge at 229 Å, provided that there is a significant helium abundance in the star's atmospheres. The temperature of the other white dwarf HZ43 has been inferred to be as high as 10^5 K, and it is detected as a very soft X-ray source. Although high for a white dwarf, such a temperature is comparable with temperatures derived for the central stars of planetary nebulae by the Zanstra method—in which the ultraviolet flux from the star is indirectly estimated by observation of optical re-emission of the energy from the ultraviolet radiation absorbed by the nebular gas surrounding the star. Thus it is reasonable that HZ43 is a fairly young white dwarf, still very hot after passing through the planetary nebula stage in which it shed its outer layers during its evolution from a giant.

The EUV observations and ultra-

violet data from the Orbiting Astronomical Observatory 2 (Holm *Astrophys. J. Lett.* **210**, L87) imply that Feige 24 is not quite so hot, around 6 to 7×10^4 K. To predict the EUV fluxes, Margon *et al.* attempt a model atmosphere calculation for a pure hydrogen atmosphere, and they find that the low observed flux shortward of 170 Å implies that there must be at least some helium present in the atmosphere to provide enough opacity shortward of the ionised helium edge. They suggest a possible, but less probable, alternative in the presence of absorption by ionised helium in the interstellar gas. Feige 24 is known from spectroscopic evidence to be a binary pair of a white dwarf and a cool main sequence dwarf showing emission lines. By subtracting the expected infrared flux of the white dwarf from the total flux of the pair, Holm deduces the energy distribution and hence a rough classification for the main sequence star. By comparison with known main sequence stars, it is then possible to infer a distance of

about 90 pc for the binary pair. For a spectral region shortward of the Lyman limit at 912 Å there is very strong absorption in the interstellar medium by bound-free transitions of neutral hydrogen. The detection of any EUV flux from Feige 24 at this distance indicates that the local interstellar hydrogen is only about 0.01 to 0.02 particles per cubic centimetre, very low compared with the mean value for the Galactic plane of about 0.6 particles per cubic centimetre. This reinforces the view that the interstellar medium is very clumpy, with strong concentrations into clouds coexisting with a tenuous intercloud medium.

It has been suggested that Feige 24 shows the spectral characteristics of old novae, in which accretion of gas from the red dwarf across onto the white dwarf has liberated radiation. If such processes are still occurring, the resulting radiation is liable to be variable, and the continued use of Feige 24 as a spectrophotometric standard might justifiably be questioned. □

of photon-atom interactions as a diagnostic method. The momentum transferred to an atom, of mass m and velocity V , by resonance absorptions, at wavelength λ , will result in a recoil through an angle α_0 ($= h/m V \lambda$) with a recoil in the opposite direction for stimulated emission. Since spontaneous emission is random in direction the steady state recoil experienced by an atom passing through a laser beam will saturate at a value of $\alpha_0 t / 2\tau$, where t is the time spent in the laser beam and τ is the natural life time of the atomic state being excited. In the experiment of Bhaskar *et al.* (*Phys. Rev. Lett.* **38**, 14; 1977) $\alpha_0 \approx 3 \times 10^{-5}$ rad per photon absorption and the net atomic beam deflection observed at 80 cm from the interaction region is several millimetres. This photon dynamical effect can now be combined with the atom recoil technique where the laser beam is perpendicular to the plane containing the atom and electron beams. This will yield the total cross section for scattering of electrons from the excited state $3^2P_{3/2}$ ($M_F = 3$) using the relation

$$\Delta I_{on} / \Delta I_{off} = f(\sigma_e / \sigma_g) - 1 + 1$$

where ΔI_{on} and ΔI_{off} are the atom scattering out signals with the laser beam on and off respectively, and σ_e and σ_g are the total cross sections for electron scattering off excited and ground state atoms respectively. Bhaskar *et al.* have measured the absolute value of σ_e at an incident electron energy of 4.4 eV and obtained a value of $(285 \pm 55) \times 10^{-16}$ cm².

Extension of this technique to differential cross-section measurements and to experiments in which the spin state of the atom is selected will greatly increase the knowledge of fundamental processes in atomic scattering. □

Effects of photon momentum

from a Correspondent

THE photon dynamics resulting from light absorption by an atom are sufficient to cause an observable deflection of the excited atom. Radiation effects of this nature can now be utilised as techniques in atomic scattering experiments opening up a new and exciting area of investigation.

The advent of commercially available tunable continuous wave (CW) dye laser systems now enables atomic physicists selectively to prepare the target atom in a scattering experiment in a well defined excited atomic state. The tunable power currently available (several milliwatts at a bandwidth of 5 MHz) is sufficient to maintain the necessary excited state population required for a scattering experiment to be performed. The sodium atom with its resonance transition 3^2S-3^2P , suitably placed in the region of efficient organic dye operation is a very attractive candidate for optical pumping. Work on the scattering of state selected sodium atoms ($3^2P_{3/2}$) off neon atoms has been reported (Carter *et al.*, *Phys. Rev. Lett.* **35**, 1144; 1975) in the determination of ground and excited potentials of the NaNe molecules. Hertel *et al.* (*J. Phys. B*, **7**, 570; 1974) were the first to perform high resolution electron scattering experiments on laser-excited sodium atoms where elastic, inelastic and super-elastic processes were studied.

In the work by Hertel only differential

cross section $\sigma(\theta)$ measurements were made where the scattered electron was monitored over a limited angular scattering range θ . To establish a total cross section, measurement of $\sigma(\theta)$ is required over the angular range $\theta = 0$ to 180° . An alternative method for directly measuring the total cross section σ for electron atom scattering, that is, atomic beam recoil, was developed several years ago (Bederson in *Methods of Experimental Physics* (ed. Bederson B. and Fite W. L.) **7A**, 89 (Academic, 1968) for measuring σ or the scattering of electrons off ground state atoms. In this procedure a well defined narrow atomic beam is cross fired at right angles by a beam of low energy electrons. The collimation in the apparatus after the interaction region is sufficient to enable the reduction in flux of the atomic beam to be monitored. The signal scattered out of the incident beam by the electrons $\Delta I = I_o - I_s$ (where I_o and I_s are the atomic beam current with the electron beam off and on respectively) therefore gives a direct measure of the total electron scattering cross section. The removal of atoms from the beam is the dynamical effect of the momentum transfer imparted by the scattered electron to the atoms and this is sufficient to cause atomic recoils of several degrees.

No scattering experiments so far have taken advantage of the dynamical effects



A hundred years ago

A MEDAL to commemorate the part taken by the Institute of France in the observation of the transit of Venus has been struck at the national mint. It bears the representation of a female passing before the car of Apollo, with the motto in Latin, "Quo distent spatio, sidera juncta docent." Each member of the Institute has received a silver medal, as well as the heads of the mission; the assistants received a bronze one. A medal has been cast in gold and presented to M. Dumas, the President of the Transit Commission. The expenses were defrayed by subscription among the members of the Institute. From *Nature* **15**, February 15, 344; 1877.

review article

Strategy for detection of cancer hazards to man†

Sir Richard Doll*

Forty-four years ago, Sir Ernest Kennaway and his colleagues identified, for the first time, a pure chemical that was capable of causing cancer in animals, and a year later isolated another from material that was widespread in the environment. At that time, and even after these crucial observations, it was commonly assumed that cancers were an inevitable accompaniment of ageing and that little could be done to reduce the mortality they caused. It is now clear, however, that most, if not all cancers have environmental causes and can in principle be prevented. The identification of environmental hazards and clarification of the mechanisms through which they cause disease are thus among the highest priorities in cancer research.

SINCE Kennaway's seminal work^{1,2}, the evidence that cancer can be prevented has accumulated steadily and is now overwhelming. Whether it will ever be possible to prevent the disease altogether, as we can now prevent poliomyelitis and scurvy, is impossible to say until we know more about the mechanism by which it is produced; but we should be able to reduce the age-specific incidence rates—which account for a quarter of all deaths of men in Britain under 75 years of age—by at least 80 to 90%. That this is so, is suggested primarily by the great variation in the incidence of different types of cancer in different communities and in different parts of the world.

This variation is illustrated in Tables 1 and 2, which show maximum and minimum incidence rates for all cancers that are common enough somewhere for the disease to affect more than 1% of men or women by 75 yr, in the absence of other causes of death. The range of variation is never less than fourfold and is sometimes more than a hundredfold. I have presented the data as cumulative incidence rates up to 75 yr, despite the fact that rates at old ages tend to be unreliable where personal medical services are sparse, to illustrate how common some cancers can become in countries like our own where half the population lives to be over that age. In estimating the range of variation, however, I have limited comparison to ages under 65 yr.

With rare cancers, the very fact of their rarity makes the demonstration of their incidence in the small populations that have been studied outside the industrialised countries extremely difficult, if not impossible. Nevertheless, some clearly vary; for example, Burkitt's lymphoma, which never affects more than 1 in 1,000 people³, varies at least 100-fold. Others, such as acute leukaemia in young adults and nephroblastoma in children, seem to be fairly constant everywhere.

Some of this variation is, of course, genetic in origin. That heredity is not the principal cause is, however, shown by migrant groups, whose experience of disease usually changes when they change their way of life in a new country, and by the variation in the incidence of cancer with time. The latter can seldom be demonstrated conclusively, partly because it is difficult to compare the

efficiency of case finding at different periods, and partly because a high standard of case registration has obtained in only a few places for more than 10 yr—the most notable being Denmark and Connecticut, USA. We have, therefore, to rely to a large extent on trends in mortality, which may also be influenced by changes in the efficacy of treatment.

Nevertheless, some changes are so gross that it is impossible to doubt that there has been a real change in incidence: for example, the increase in lung cancer in all developed countries, the increase in oesophageal cancer in the black population of South Africa, and the decrease in gastric cancer in the USA. Many lesser changes have taken place in Britain during the last 20 years. These are shown in Table 3, which lists all those cancers for which the mortality rate, standardised for age, has changed by more than one per cent a year between 1958 and 1973. Several of these changes cannot be dismissed as artefacts, because the disease is easy to diagnose and the death rate has increased despite improving treatment (as with melanoma and cancer of the testis). Taken altogether, the evidence suggests that all common cancers have varied in incidence from time to time, just as they now vary from place to place.

Much of this variation can now be attributed to the action of specific agents. Indeed, so many are now known that we may reasonably hope to extrapolate from past experience in seeking others. I shall, therefore, begin by reviewing briefly the known agents and the ways in which they have been detected.

Iatrogenic hazards

About a score have been prescribed by doctors (Table 4). Most have caused only a few cases and their effect has been apparent to clinicians and pathologists because the microscopic appearance of the tumour or the site in which it occurred was so unusual. Examples include adenocarcinoma of the vagina in young women whose mothers had taken stilboestrol during the relevant pregnancy (recognised when a lift broke down in Boston and the paths of a gynaecologist and a pathologist crossed for sufficiently long for them to exchange experiences); angiosarcoma of the liver and spleen following the injection of thorotrast; squamous carcinoma of the thigh following the local application of coal tar ointment; squamous carcinoma of the

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†This article is based on the Sir Ernest Kennaway Lecture delivered at the Royal Institution on 11 November 1976.

Table 1 Range of variation in the incidence of common cancers in men (unless specified ♀)

Type of cancer	High incidence area	Cumulative risk by 75 years of age* (%)	Range of variation†	Low incidence area
Skin	Australia, Queensland	> 20	> 200	India, Bombay
Oesophagus	Iran, NE	20	300	Nigeria
Bronchus	England	11	35	Nigeria
Stomach	Japan	11	25	Uganda
Cervix uteri ♀	Colombia	10	15	Israel (Jewish)
Liver	Mozambique	8	70	Norway
Prostate	USA (black)	7	30	Japan
Breast ♀	USA, Connecticut	7	5	Uganda

*In absence of other causes of death.

†At ages 35–64 years.

palm of the hands associated with arsenicism; reticulo-sarcoma of the brain following immunosuppression to prevent rejection of a renal transplant; and a peculiar type of liver tumour in women using steroid contraceptives.

The only known agents in this group that have caused any substantial number of cancers have been ionising radiations, which probably caused between 5 and 10% of all childhood cancers in Western Europe and North America during the 1950s and 60s, when used for diagnosis during the pregnancy of the mother; and oestrogens, prescribed for post-menopause women, which may account for much of the recent increase in the incidence of endometrial cancer in the USA. In both cases, the risks were overlooked for a long time, because the tumours did not differ in any obvious way from the tumours that occurred quite commonly as a result of other causes, and they were eventually detected only after large scale case-control studies had been carried out with the object of investigating the role of the agents in the aetiology of the particular disease⁴⁻⁶.

Despite the number of tumours caused by these last two agents, the total number of cases that are iatrogenic in origin is much less than readers of Illich's *Medical Nemesis* might suspect and cannot have constituted more than a minute proportion of the total impact of cancer on society.

Occupational hazards

Other agents have caused hazards in a wide variety of occupations. These are listed in Tables 5 and 6. In three instances the risks were discovered after the agents (4-aminobiphenyl, mustard gas and vinyl chloride) had been shown to cause cancer in animals. Other agents were discovered incidentally in the course of investigating other industrial hazards, as in the case of cancer of the prostate in cadmium workers and cancer of the lung in rubber workers. Most, however, were discovered because the interest of a clinician, an epidemiologist, or a pathologist was aroused by the observation of a cluster of cases that seemed too large to be easily attributable to chance. Sometimes

these clusters have been quite small, as when Dr John Jones reported that he was disquieted to have seen two employees of the Mond Nickel Company develop nasal sinus cancer within a year. Often, however, the risk has been overlooked for a long time, particularly when the same type of cancer was common in the general population as a result of other causes.

Like the iatrogenic hazards, these occupational ones cannot have been responsible for more than a very small proportion of all cancers, as the total number of men who have been exposed in the course of their work, other than open air workers exposed to ultraviolet light and, to a lesser extent, the wide variety of workers exposed to asbestos, is small in proportion to the population as a whole. They are important, however, for two reasons.

First, they are important to the workers concerned who have had a 50% risk of developing the disease in some industries. Indeed, in one small group of 19 men employed on distilling 2-naphthylamine, the risk proved to be 100%, 18 dying of bladder cancer and the last having been killed in an accident shortly after the disease was diagnosed. Second, some of the agents concerned have found their way into the general environment, so that millions of people have been exposed to them unintentionally and sometimes in an uncontrolled way. These agents are listed in Table 6. It is easy enough to dismiss the corresponding risks on the grounds that the doses are minute; but we can no longer assume that thresholds exist for chemical or physical agents and we ought neither to ignore nor to condemn them until we have derived quantitative relationships between the dose to which individuals are exposed and the resultant incidence of the disease. At present we can do this only very crudely. Nevertheless, any quantitative evidence is better than none.

Industrial pollution

Consider, for example, polycyclic hydrocarbons and asbestos. For the first, we have evidence that the large amount of benzo(a)pyrene inspired by gas retort house workers produced a risk of lung cancer only about 80%

Table 2 Range of variation in the incidence of common cancers in men (unless specified ♀)

Type of cancer	High incidence area	Cumulative risk by 75 years of age* (%)	Range of variation†	Low incidence area
Colon	USA, Connecticut	3	10	Nigeria
Buccal cavity	India, part	> 2	> 25	Denmark
Rectum	Denmark	2	20	Nigeria
Bladder	USA, Connecticut	2	4	Japan
Ovary ♀	Denmark	2	8	Japan
Corpus uteri ♀	USA, Connecticut	2	10	Japan
Nasopharynx	Singapore (Chinese)	2	40	England
Pancreas	New Zealand (Maori)	2	5	Uganda
Penis	Uganda, part	1	300	Israel (Jewish)

*In absence of other causes of death.

†At ages 35–64 yr.

greater than the national average⁸. Since the residents of large towns are unlikely to have been exposed to more than one-hundredth of that amount—mainly from the combustion of domestic coal⁹—the contribution of these agents to the urban excess of the disease is unlikely to have been large, a conclusion that is confirmed by the low incidence of the disease in non-smokers irrespective of where they live.

As for asbestos, we know that it reaches the ambient air from a variety of sources, including the clothes of asbestos workers. These were presumably the principal source of exposure of the 37 men and women in nine countries who are reported to have developed mesothelioma of the pleura after being household contacts of asbestos workers¹⁰. The maximum concentration that has been found in the air near building sites where asbestos was being sprayed is, however, three orders of magnitude less than that which has been

Table 3 Changing mortality rates from different cancers* (England and Wales, 1958 to 1973)

Type of cancer	(% Change	
	Males	Females
Melanoma	+95	+47
Myelomatosis	+65	+70
Lung	+29	+94
Pancreas	+19	+20
Testis	+19	
Oesophagus	+16	+17
Stomach	-25	-34
Buccal cavity	-38	-24
Cervix		-23

*All cancers with rate of change equal to or more than 1 % per year.

regarded as an acceptable concentration in the asbestos industry¹¹, and the amount that is commonly present in town air is still less by another two orders of magnitude. Unfortunately, we are still uncertain about the size of the risk that is associated with this accepted concentration of about 0.1 mg m^{-3} . In one study of an asbestos textile factory in England¹², it was found that the relative risk of lung cancer for men who had been employed for 20 yr decreased progressively from 10 times 'normal', if they had been employed for at least 10 years before 1933 (that is, the date when some control of asbestos dust in the air of factories first became effective in the UK), to $3\frac{1}{2}$ times normal if they had been employed before 1933, but for less than 10 years, to $1\frac{1}{2}$ times normal if they had first been employed only after 1933. Few men who were first employed only after 1951 have yet been employed for 20 yr, but preliminary estimates (which are highly unreliable because the number of men observed is so small) suggest that some risk may still have persisted since that date. Detailed dust records were not obtained in the early years in a way that enables them to be compared with present data, but the amount of dust in the air was initially gross and continued to be much greater than at present for some time after 1933. Even in 1951, the mean dust level to which men were exposed is likely to have been $3\frac{1}{2}$ times greater than it is now, and we shall have to wait for many years before industrial data enable us to make a reasonable estimate of the possible risk associated with an average exposure up to 0.1 mg m^{-3} during normal working life.

Measurements like these exculpate individually as major contributors to the gross variation in cancer incidence between countries all the agents that are known to cause specific occupational hazards (other than ultraviolet light), whether acting within industry or as pollutants in the general environment. In so far as we can explain this variation, it seems rather to be due to differences in social behaviour, diet and the opportunity for infection.

Table 4 Iatrogenic causes of cancer

Agent	Site of cancer
Diagnostic or therapeutic X rays	All sites
Thorium	Bone
Thorotrast	Liver, spleen marrow (leukaemia)
Polycyclic hydrocarbons in coal tar ointments	Skin
Alkylating agents	
Melphalan, cyclophosphamide	Marrow (leukaemia)
Oestrogens	Endometrium, breast (M) ? breast (F)
Stilboestrol (transplacental)	Vagina
Steroid contraceptives	Liver
Androgens (anabolic steroids)	Liver
Arsenic	Skin, lung
Chlornaphazine	Bladder
Immunosuppressive drugs	Reticulosarcoma
Phenacetin	?Renal pelvis

Other hazards

The other agents that are known to cause cancer are listed in Table 7. The number is small, but the cancers that have been produced are legion. Cancers of the buccal cavity attributable to chewing, for example, account for a quarter of all cancers in men in parts of India, while cancers of the lung attributable to smoking account for more than a third of all lethal cancers in men in Britain.

One agent, aflatoxin, was discovered to be a powerful carcinogen as a result of an outbreak of poisoning that killed 100,000 ducks and turkeys in British farms. The outbreak was traced to a consignment of peanut meal contaminated with *Aspergillus flavus* which produced a metabolite that not only caused acute liver failure in poultry, but also caused cancer of the liver in minute doses in a wide variety of animals^{13,14}. Human liver cells contain the enzymes necessary to produce the active agents (the epoxy-metabolites of aflatoxin), and *Aspergillus flavus* is a frequent contaminant of foodstuffs stored under hot and humid conditions. Now it has been shown that the incidence of liver cancer in Thailand, Singapore, Kenya, Swaziland and Mozambique is proportional to the amount of aflatoxin in the diet^{15,16}. The vagaries of daily diet make it unlikely that we shall ever be able to establish the relationship in individuals; but the total evidence is strong enough to justify an attempt to prevent the disease by reducing exposure. Unfortunately, this will not be easy, as fungal contamination is difficult to avoid under the conditions in which the staple foods are commonly stored in the tropics, and it will be expensive to substitute alternative methods.

The other agents listed in Table 7 were, for the most part, discovered as a result of clinical acumen dating back

Table 5 Occupational causes of cancer not contributing to general environmental pollution

Agent	Site of cancer
Ultraviolet light	Skin
Aromatic amines	Bladder
2-naphthylamine	
1-naphthylamine	
Benzidine	
4-aminobiphenyl	
bis-chloromethyl ether	Bronchus
Benzene	Marrow (leukaemia)
Mustard gas	Bronchus
	Larynx
	Nasal sinuses
(Nickel ore)	Bronchus
	Nasal sinuses
(Chrome ore)	Bronchus
Cadmium (?)	Prostate
Agents in isopropyl oil	
hardwood furniture manufacture	Nasal sinuses
leather goods manufacture	

Table 6 Occupational causes of cancer contributing to general environmental pollution

Agent	Site of cancer
Ionizing radiations	Bronchus Skin Bone Marrow (leukaemia)
Polycyclic hydrocarbons in soot, tar, and oil	Skin, scrotum Bronchus
Arsenic	Skin Bronchus
Asbestos	Bronchus Pleura, peritoneum
Vinyl chloride	Liver (angiosarcoma)

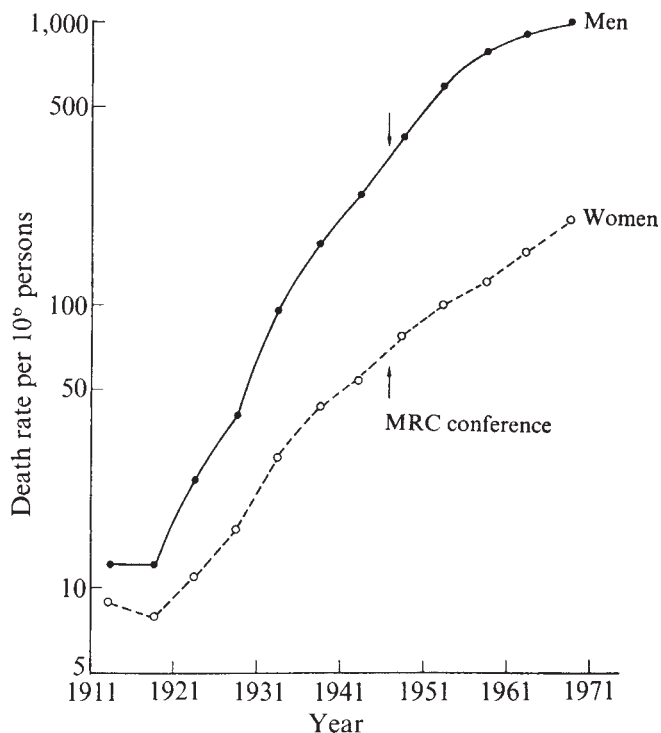
nearly 200 years to Soemmerung's description of lip cancers in pipe smokers¹⁷. Clinical associations, however, tend to be overlooked if the conclusions suggested are socially unacceptable, and little attention was paid to the possible effects of smoking until the death rate from lung cancer in men had increased 25-fold (Fig. 1). The conference that was called by the Medical Research Council in 1947 could not decide whether the increase was real or an artefact due to improved diagnosis, but it did recommend a study to seek a possible cause. The subsequent enquiry showed that patients with lung cancer tended to smoke more than other patients, and within 3 years it was possible to conclude from the human evidence alone that cigarette smoking was a cause of the disease¹⁸. Whether these observations, or any others that are both practicable and ethical to obtain, can be regarded as constituting logical proof that a carcinogenic agent has been detected is debatable and is not of great interest. What is of interest is whether the evidence justifies an attempt at prevention and, if so, whether removal of the agent is followed by the result we seek¹⁹.

Prevention should, of course, ideally be carried out in a controlled way with random allocation of individuals, or groups of individuals, to experimental and control series. In practice, however, this is seldom possible as the experiment is likely to require the cooperation of a large number of people who have to be convinced that it will be successful before it can be begun. Everyone, therefore, will want to be in the experimental group. This is what has always happened in industry; but when the disease disappears, as so many occupational cancers have, few people have challenged the logic that led to the intervention.

Once smoking began to be studied seriously as a cause of cancer, data were soon collected that confirmed the old clinical observation of a relationship with cancer of the mouth, and there are now strong grounds for believing that it also causes many cancers of the pharynx, larynx and oesophagus. Weaker, but consistent, evidence—like that summarised in Table 8—also suggests that it may play a part in producing cancers of the bladder and pancreas.

That alcohol contributes to the causation of a large proportion of cases of cancer of the oesophagus, and to a somewhat smaller proportion of cancers of the mouth, pharynx and larynx, in some countries, has been suspected ever since the mortality from these cancers was found to be unusually high in publicans, waiters and others employed in alcoholic trades²⁴. The subject has, however, proved so

unattractive to research workers that we still do not even know whether it is the alcohol itself that is responsible (possibly by solubilising a specific agent) or whether it is another component of alcoholic drinks that may vary in amount from one drink to another. That the subject has been unpopular may have been because pure alcohol is not carcinogenic to laboratory animals or because the amount consumed by the individual is difficult to assess with accuracy—or even because of a natural aversion for investigating such an unpopular subject. Interest in it has, however, been revived and studies are now being carried out on oesophageal cancer in parts of France where the characteristic alcoholic drinks are cider and cider-based liqueurs²⁵. If, however, alcoholic drinks are important in the aetiology of cancers of the upper digestive tract in Europe and North America, they certainly do not explain

**Fig. 1** Trend in crude death rate from lung cancer 1911–1971, by sex, showing state at time of MRC conference.

the astronomical figures for the incidence of the disease in central Asia, where very little alcohol is consumed.

Viruses

Another potential hazard is infection with an oncogenic virus, but it is still an open question whether viruses can ever cause cancer in man. Direct evidence of case-to-case infection is extremely weak—no-one, for example, who has used adequate controls has been able to repeat Vianna, Greenwald and Davies's²⁶ observation that patients with Hodgkin's disease have had unusually close personal contact—and the laboratory evidence that cancers are associated with viral infection is open to several interpretations.

Table 7 Other environmental causes of cancer

'Agent'	Site of cancer
Sunlight	Exposed skin (rodent ulcer, squamous carcinoma, ? melanoma)
Associated with use of 'Kangri' and 'dhoti'	Skin of abdomen, groin, and thigh (squamous carcinoma)
'Reverse smoking'	Palate
Chewing betel, tobacco, lime	Mouth
Smoking	Mouth, pharynx, larynx, bronchus, oesophagus, bladder, ? pancreas
Aflatoxin	Liver
Schistosomiasis	Bladder
Associated with sexual intercourse	Cervix uteri

Table 8 Risk of death from cancer of pancreas in continuing cigarette smokers relative to life-long non-smokers (prospective studies)

Population*	Sex	Cigarette smokers	Risk compared with that in non-smokers			
			1-9	Current cigarette smokers smoking per day 10-20	21-39	40 or more
US citizens (77)	F	1.8				
(108)	M	2.7				
US veterans (493)	M		0.9	1.9	2.2	1.9
Canadian pensioners (89)	M		1.4	2.0	2.4	
Swedish random sample (46)	M		1.6	3.4	5.9	
British doctors (92)	M		1.4	1.4	2.1	

*Numbers of deaths in parentheses: for details see Hammond²⁰, Kahn²¹, Department of National Health and Welfare²², Cederlof *et al.*²³ and Doll and Peto¹⁹.

Consider, for example, the extensive evidence that now connects the EB virus with Burkitt's lymphoma and carcinoma of the nasopharynx. In epidemic areas these diseases occur nearly always in individuals infected by the virus. Viral DNA is present in all the tumour cells and determines the expression in them of virus-coded neoantigens; and virus production can be activated in some of the tumour cells in the laboratory. The EB virus is widespread in human society and is the cause of infectious mononucleosis, when it stimulates the proliferation of mononuclear cells of the lymphatic series. *In vitro*, it confers the property of continuous growth on normal human B lymphocytes in culture, in a way that is analogous to malignant transformation. Finally, it has been shown to cause malignant lymphomas in South American cotton top marmosets on experimental inoculation.

What more can laboratory investigation be expected to do? The International Agency for Research on Cancer²⁵ is attempting to relate the development of the disease to new infection by following up children in the West Nile district of Uganda from whom blood samples have been taken for serum studies; but the results are unlikely to be decisive if, as one suspects, viral infection is only one of several conditions necessary for the development of the disease. If this turns out to be so, the only remaining approach will be to develop a vaccine that can be shown to prevent the disease²⁷—immensely difficult though that must be—and the same would also be true if cervix cancer were firmly linked to infection with the type II herpes simplex virus or to any other infective agent.

That cervix cancer is venereal in origin is now virtually certain. We know that the disease spares nuns and is most common in prostitutes; that the risk increases with the number of marriages and with the age at which coitus first occurs, but not with the number of pregnancies, nor with the frequency of intercourse within marriage; and that more of the husbands of affected women have had extra-marital intercourse than of the husbands of control women²⁸⁻³⁰. To these facts we can add Beral's observation³¹ that the mortality from cervix cancer in cohorts of women of different ages varies with the incidence of gonorrhoea at the time they were 20 years old, and the accumulating evidence that obstructive methods of contraception are protective³².

Interaction of agents

If viruses do, in fact, cause cancer, they may do so only by interacting with other factors, and this may account for the separate correlation of hepatitis B antigen and aflatoxin with hepatoma and of the EB virus and gross malarial infection with Burkitt's lymphoma. That two different agents may interact to produce cancer has been established

by occupational studies which have demonstrated interactions between smoking and asbestos and smoking and ionising radiations and have provided quantitative data like those on the incidence of lung cancer in uranium miners in the USA³³.

Similar data are now also being obtained by the International Agency for Research on Cancer²⁵ for the interaction between tobacco and alcohol in the production of oesophageal cancer in France. Preliminary estimates, based on a retrospective case-control study of patients and a random sample of the population, suggest that the risk of developing the disease increases both with the amount smoked and with the amount drunk, until among men who drink 81 or more grams of ethyl alcohol (equivalent to 7 whiskies) and smoke 20 or more cigarettes a day, the risk is 45 times that in those continent men who drink less than 40 grams and smoke less than 10 cigarettes a day.

The existence of such interactions, which has been suspected since the early experiments of Rous and Kidd³⁴ and Berenblum and Shubik³⁵, can be of great importance. For they may provide not only the explanations for apparently conflicting observations, but also alternative means of preventing disease, one of which may be more practicable than the other. If, for example, smoking and asbestos interact to produce bronchial cancer, there may be no point in trying to give quantitative labels to their respective shares. Both may be responsible for producing more than half the cases in the sense that the elimination of either would reduce the total risk by more than 50%. The important conclusion is that we can break the chain of causation at either of two links, and it may be as idle to try and partition responsibilities as it is to try to quantify the relative contributions of nature and nurture to disease in general.

Strategy for detection

In this incomplete review, I have sought to provide an account of the hazards of cancer to man and to use the experience of the past to indicate how other hazards can be detected in the future. We cannot, of course, hope to detect hazards efficiently until we know how cancer is produced, so that a policy for detection must include the support of basic biological research. Success in this field is dependent on the development of ideas and is difficult to foster except by providing the conditions in which outstanding investigators are able to give full rein to their imagination.

Test of products before use

Two types of hazard stand out as socially unacceptable, although they are relatively unimportant numerically: that is, the hazards associated with occupation and the use of prescribed drugs. The evidence that chemical carcino-

gens, or their reactive metabolites, are commonly electrophilic, react with DNA and, in consequence, are able to induce mutations, has suggested that it may be possible to predict carcinogenicity by relatively quick and simple tests with bacteria. It is not certain that any one test, or combination of tests, will prove to be adequate, but preliminary results are encouraging³⁶.

This, however, will not absolve us from making arrangements to detect hazards to man. Not only must we expect some chemicals to escape the net, particularly if they are hormones or act as promoters, enzyme inducers, or repair inhibitors; but we shall also wish to use some, and possibly many, substances that give positive results in animals, if the benefits are judged to be great enough to outweigh the harm. The continuing challenge to laboratory scientists is to discover enough about metabolism and the mechanisms of carcinogenesis in man to enable us to predict the extent of the hazard if the substance is used in a particular way. Meanwhile, we must either refrain from introducing such immensely useful materials as phenobarbitone, isoniazid, DDT, saccharin, and the minute amounts of stilboestrol that are used to fatten cattle, although they do not have any detectable effect in man, or arrange to detect and quantify hazards to man at the earliest opportunity.

Record linkage

Embryo mechanisms suitable for this purpose already exist in Britain, that are based on the records of the National Health Service and the Office of Population Censuses and Surveys, and to a varying extent in other countries³⁷⁻⁴⁰. Identifying information about all people who develop cancer is already integrated into a single file in England and Wales, and we now need to create similar files of people who are exposed to different chemicals. The Registrar-General has begun to do this by maintaining a file of a 1% sample of the population which is up-dated at each successive census in respect of births, deaths, immigrants and emigrants, and by recording the cumulative occupational experience of the selected sample throughout their lives. This file can be linked within OPCS to the occurrence of cancer and so can provide an automatic assessment of the risks associated with any particular industry. It will be necessary, however, to increase the size of the sample to at least 10% before it will be of any

value for any but the larger risks. Very little information is included about specific exposures and it will probably also be necessary to require industry to maintain records of all employees who are exposed to new chemicals, so that they can be checked periodically against the file of cancer cases. Similarly, if we are serious in our desire to control adverse reactions to drugs, including reactions like cancer which occur only infrequently or after a prolonged latent period, we shall have to introduce some system for recording drug usage such as Skegg⁴⁰ is now doing on a pilot scale in 20 practices, or possibly create a new category of drugs that can be prescribed only on special forms for a period after they are introduced. Data that identify the patients can then be stored in a computer for subsequent matching with similar data in the cancer index.

The concept of linking records by computer has given rise to anxiety lest they are used to the detriment of the individual, as has happened when personal records have been linked by credit organisations in the USA, and some doctors have been worried by the apparent loss of confidentiality. Confidentiality can, however, be protected in a computer much more easily than in the standard case-note, and I know of no instance where the provision of personal information to a bona fide medical research worker has been abused. It is, of course, for the public to decide; but in my experience, most people understand that we cannot protect them against disease unless we are allowed the necessary tools. Record linkage of the type required is, moreover, not expensive when the essential records have to be made for other purposes, as they now all are—the only defect of the present system being that the records are not organised in a way that enables them to be used to detect hazards to health.

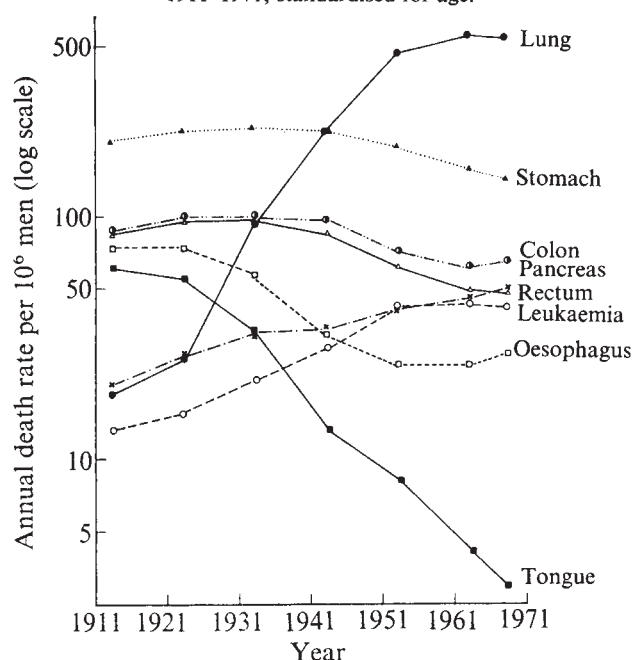
Cancer incidence and mortality rates

Record linkage is less useful as a means of detecting hazards arising from environmental pollution or household materials, if there is no greater human exposure at the source of their creation or manufacture. In these circumstances we can rely only on cancer and death registries to arouse suspicion by revealing changes in the incidence of disease with time or place. Such registries are, also, one of our principal weapons for obtaining clues to other hazards that are not iatrogenic, occupational or attributable to pollution and which account for the mass of cancers that fill our hospitals today. Death rates for most of these and other cancers that were common between 1911 and 1970 are shown in Figs 2 and 3. The rates have been standardised for age and are limited to ages under 65 years, as diagnostic services for old people were so much less effective in the earlier part of the period. All the trends must have been affected by changing diagnostic standards and many will also have been affected by improving treatment, but we cannot avoid the conclusion, which accords with clinical experience, that cancers of the stomach, colon and rectum, and cancers of the breast and uterus (though not of the ovaries) have been common for many years so that if they are environmental in origin, as the evidence of geographical and social variation suggests, the responsible factors must have been prevalent at least before the beginning of the century.

Investigation of diet

One feature of the British way of life that has distinguished it from the Asiatic or African way for the appropriate length of time is its diet, and factors related to diet have been suspected of contributing to the production of cancer for many years. Until recently, however, there has been little precise evidence and very few promising ideas. Now, a spate of fresh ideas enables dietary factors to compete for the interest of research workers on almost equal terms with

Fig. 2 Trends in mortality from common cancers in men 1911–1971, standardised for age.



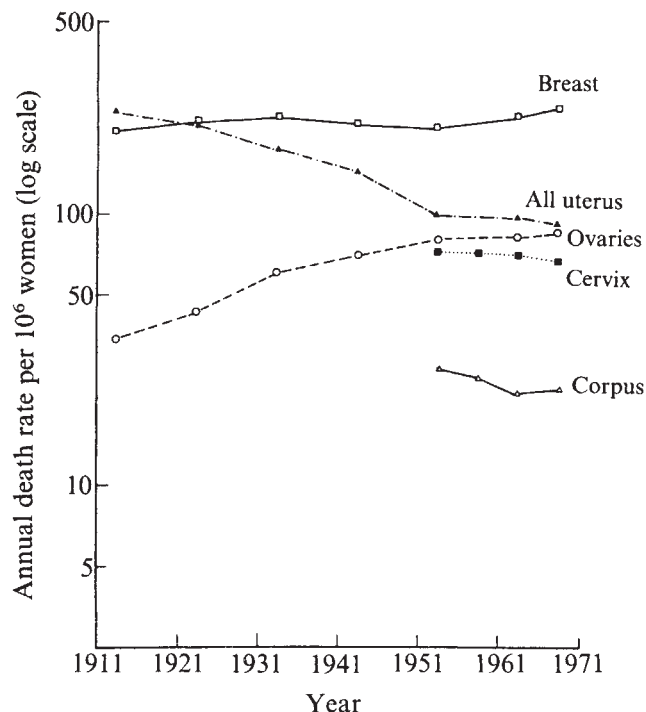


Fig. 3 Trends in mortality from common cancers of breast and genital tract in women, 1911–1971, standardised for age.

viruses and immune reactions. One such idea, to which I referred previously, was that aflatoxin might be responsible for tropical liver cancer. Another was the discovery that the cycad nut contained a constituent (cycasin) that was harmless in itself, but which gave rise to a carcinogen in the bowel of an animal with a normal intestinal flora. This was not fruitful in itself, but it focused interest on the possible role of intestinal bacteria and led Williams to examine the intestinal flora of people living in areas where the incidence rates of colon cancer were grossly different. Populations with a high risk were found to be characterised by the presence in their faeces of large numbers of anaerobic bacteria that were able to dehydrogenate bile acids, and Williams and his colleagues postulated that this might result in the production of carcinogens that acted locally on the colonic mucosa⁴¹. In 1975, Meade and his colleagues at Northwick Park, working in conjunction with Williams' group, reported the preliminary results of a retrospective study of patients with and without large bowel cancer which supported this idea⁴², and these findings have now been confirmed in twice as many patients⁴³. The original hypothesis will, however, have to be elaborated if it is to account for the low incidence of the disease in Finland and the increased incidence in the upper socio-economic classes in Hong Kong^{43,44}; and Hill⁴⁵ suggests that it may also be necessary to have a high level of vitamin K to act as a hydrogen acceptor, before carcinogens can be formed under anaerobic conditions in the gut.

Burkitt's⁴⁶ hypothesis, that the relative lack of indigestible fibre in the diet of industrialised countries might be the primary cause of a variety of diseases of the large bowel that are absent in populations whose diet consists of natural unprocessed foods has also opened up new lines of thought. The greater bulk of faeces and the more rapid transit time associated with a high fibre diet could hardly account in physicochemical terms for the sort of gross differences in the incidence of large bowel cancer that occur between black African and the English-speaking countries; but the altered conditions in the bowel might perhaps account for the variation in the intestinal flora.

A fourth way in which diet may affect the incidence of cancer is by providing the raw materials from which

nitrosamines are produced in the stomach—a group of powerful carcinogens that are still looking for human cancers to induce. Potential carcinogens of this group can be formed in acid gastric juice by the action of nitrites on secondary amines in food—the nitrites being present because of their use as preservatives, or because they were formed from nitrates in the mouth as a result of bacterial metabolism. This, however, is speculative. All we know for certain is that gastric cancer was common in the relatively poor populations of northern Europe and Japan (though not in most of the even poorer populations of Africa), that it has been becoming progressively less common in Western Europe and much less common in the USA, that it is less common in Japanese migrants to California and Hawaii than in the Japanese in Japan, and that the disease in the migrants tends to occur in those who eat pickled vegetables and dried and salted fish and not in those who eat such Western vegetables as tomatoes, celery, corn, lettuce and onion⁴⁶. Could this be, as Weisburger and Wynder⁴⁷ suggest, because the small amounts of vitamin C inhibit the formation of nitrosamines in the stomach? Alternatively, it may be that the progressive reduction in incidence is due to better preservation of food, with a consequent reduction in the opportunity for bacterial or fungal contamination.

That vitamins may play a part in protecting against cancer is also suggested by experiments⁴⁸ on animals in which the incidence of cancer was reduced by a vitamin A-enriched diet. Now two sets of human data—one a prospective study of men with known dietary histories⁴⁹ and another a biochemical investigation of serum A levels in patients and controls⁵⁰, suggest that a deficiency of vitamin A may increase the risk of squamous cell carcinoma of the bronchus in cigarette smokers.

Fat also is a possible factor. Its consumption is closely correlated with the incidence of colon cancer ($r=0.78$) and breast cancer ($r=0.79$) in different countries, and several investigators have suggested that its content in the diet may directly affect the incidence of these diseases^{51,52}. Armstrong⁵³ recently pointed out that the incidence of endometrial cancer was even more closely correlated with dietary fat ($r=0.85$) and showed that excess consumption of fat could account for five of the clinical characteristics that tend to be associated with the development of this disease; that is, obesity, early menarche, late menopause, maturity onset diabetes, and hypertension. Virtually all oestrogen produced in post-menopausal women is derived from oestrone which, in turn, is produced by aromatisation of the androstenedione secreted by the adrenals. Fat could play a part either because androstenedione is converted to oestrone in adipose tissue⁵⁴ or, perhaps, by inducing the oxidase systems that metabolise pre-carcinogens.

Conclusion

I have concluded by referring to some of the hazards that may be associated with diet, not because I believe that their existence has been proved, but because they provide an indication of the way in which the hazards that are responsible for the majority of human cancers may be detected in the future; that is, by a combination of epidemiological and laboratory enquiries. Such studies were often conducted independently in the past, with very little contact between the research workers involved. The position was, however, transformed when the World Health Organization set up the International Agency for Research on Cancer with the explicit purpose of encouraging such collaboration internationally, and it has been further strengthened in this country now that the Cancer Research Campaign, the Imperial Cancer Research Fund and the Department of Health and Social Security have joined the Medical Research Council in establishing academic units of cancer epidemiology within, or in close association with, the major departments of basic cancer research. With this collabora-

tion, a rational use of medical records, and continued support for general biological research, I see no reason why causes for the remaining common cancers should not be detected within one or two decades. That is not to say that it will be easy to prevent the disease. For if, as I suspect, these hazards are associated with the common diet of developed countries, the problems that we are now having to face in preventing tobacco-induced cancer will seem childishly simple.

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articles

Rapidly-formed ferromanganese deposit from the eastern Pacific Hess Deep

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A thick ferromanganese deposit encrusting fresh basaltic glass has been dredged from the Hess Deep in the eastern Pacific. Contiguous layers within the Fe-Mn crust have been analysed for uranium-series isotopes and metal contents. The rate of accumulation of the deposit, based on the decline of uranium-unsupported ²³⁰Th, is calculated to be approximately 50 mm per 10⁶ yr. Based on hydration-rind dating of the underlying glass and an 'exposure age' calculation, this rate is concluded to be too slow, and an accretion rate on the order of 1 mm per 10³ yr is more consistent with our data.

INDURATED metal-rich crusts associated with probable hydrothermal areas on the seafloor have been reported¹⁻⁶. Ferromanganese encrustations, sampled on active spreading centres, include those from the Mid-Atlantic Ridge, sampled during the Trans-Atlantic Geotraverse (TAG) expedition^{2,5} and during the FAMOUS project⁴, from the flank of a seamount at the crest of the East Pacific Rise¹, and from the Galapagos Spreading Center⁶. These deposits have been attributed to precipitation from hydrothermal fluids which gained high metal contents during interaction of seawater with basaltic rock at elevated temperatures. Although not conclusive, this type of genesis is more consistent with the geochemical and geological evidence than is a diagenetic formation by metal remobilisation and subsequent precipitation within sediment or formation by direct precipitation from seawater. Experimental evidence^{7,8} showing that seawater becomes greatly enriched in Fe and Mn during interaction with fresh basalt at elevated temperatures seems to support the hydrothermal origin.

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Table 1 Chemical composition of crust from station 1052 compared with other reported hydrothermal deposits and an average Pacific manganese nodule

Sample	Location	Fe (% by weight)	Mn	Ni	Co (p.p.m.)	Cu	Zn	Fe/Mn
1052 (0–3 mm)	Hess Deep	21.8	16.7	1,950	508	735	543	1.30
D6 (0–3 mm) (data from ref. 6)	Galapagos Spreading Center	0.016	52.7	100	<23	43	105	0.0003
T3-72D 253-13-21 (0–5 mm) (from ref. 2)	Mid-Atlantic Ridge (~26°N)	0.011	39.2	100	18	12	—	0.0003
AMPH D2 (from ref. 3)	Flank of East Pacific Rise	32.5	1.94	400	35	74	—	16.7
Average nodule	Pacific	14.3	19.7	7,220	3,810	3,660	—	0.72

These ferromanganese deposits are characterised by low Th contents, high apparent rates of accumulation, and immediate proximity to sources of submarine volcanism. Samples from the TAG area^{2,5} and the Galapagos Spreading Center⁶ are further characterised by extremely low Fe and high Mn contents. Conversely, other suggested hydrothermal deposits are extremely Fe-rich and very low in Mn. The AMPH D2 deposit from a seamount on the flank of the East Pacific Rise, for example, has a Fe/Mn ratio of 16.7 (ref. 3). Unconsolidated metalliferous sediment such as that found along the crest of the East Pacific Rise generally have Fe/Mn ratios close to 3.0. Bonatti⁹ suggests that the wide range of Fe/Mn ratios in these hydrothermal deposits is probably caused by drastic fractionation of the two elements, both before and after discharge of the metal-bearing solutions on to the sea floor. We report here on the geochemistry of a ferromanganese crust which is compositionally intermediate between the Mn-rich^{2,5,6} and Fe-rich hydrothermal deposits^{3,9}. We present evidence to demonstrate that this deposit must have formed at a substantially higher rate than deep-sea manganese nodules which are similar in their Fe/Mn ratios.

Our sample was collected at station 1052, on cruise 14 of the

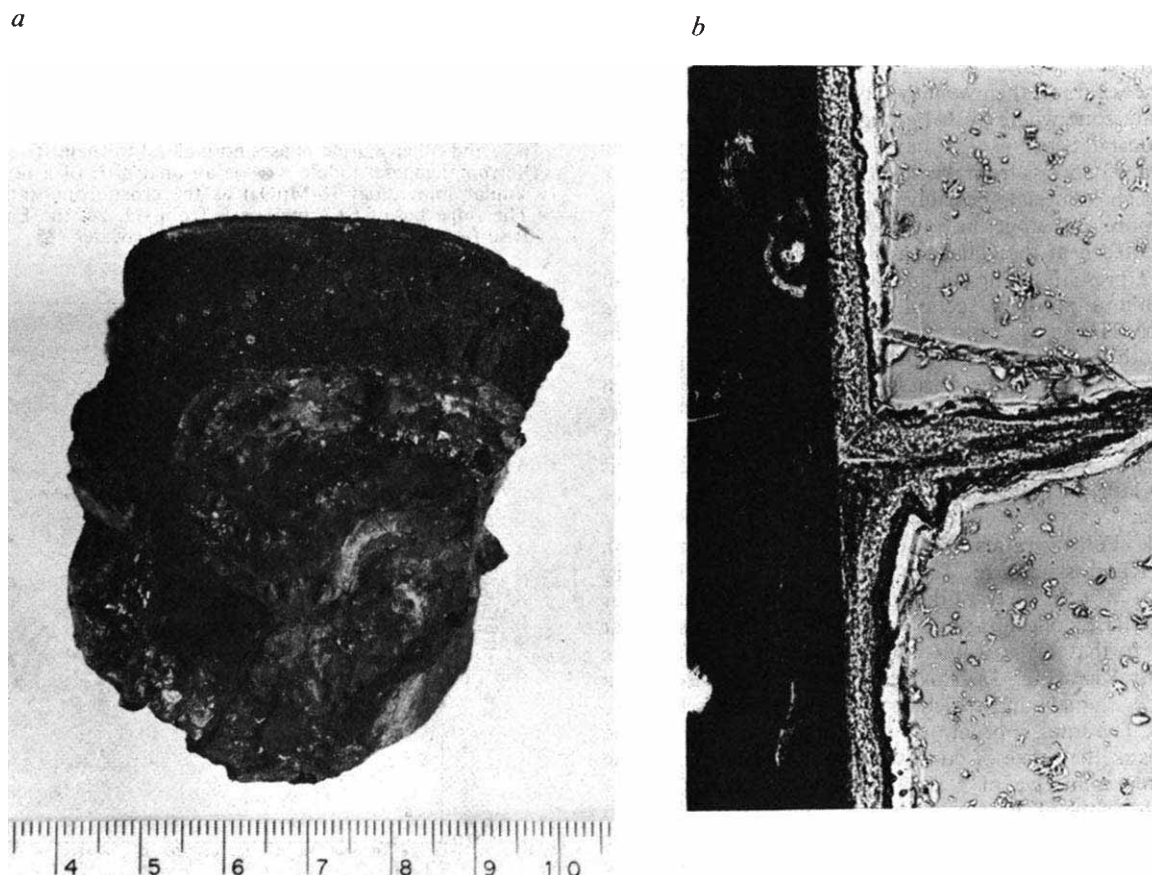
USSR RV Dmitry Mendeleev, at a depth of 3150 m, at 2° 17.8'N and 101° 1.5'W. The station is located in the axis of the Galapagos Spreading Center, just east of its junction with the East Pacific Rise. This area is known as Hess Deep¹⁰ because of its relatively great depth for an oceanic rise system.

Chemical composition

The sample which we analysed consisted of 16 mm of ferromanganese material encrusting fresh basaltic glass (Fig. 1). Table 1 shows the chemical composition of the crust from station 1052 as compared to other reported hydrothermal deposits and to an 'average' Pacific manganese nodule. Analyses were performed by instrumental neutron activation analysis except for Cu and Ni which were determined by atomic absorption. Microprobe analyses of the underlying glass reveal typical ocean theolitic composition with low alkalis and a SiO₂ content of about 50%.

This sample is unlike the Mn-rich deposits from the TAG area and the Galapagos Spreading Center, having a much higher Fe and trace metal content. It is not as Fe-rich, however, as the iron deposit AMPH D2 from the flank of the East Pacific

Fig. 1 Photograph and microphotograph of thin section cut from sample 1052. Scale in (a) is in cm; the width of (b) is equivalent to approximately 1 mm.



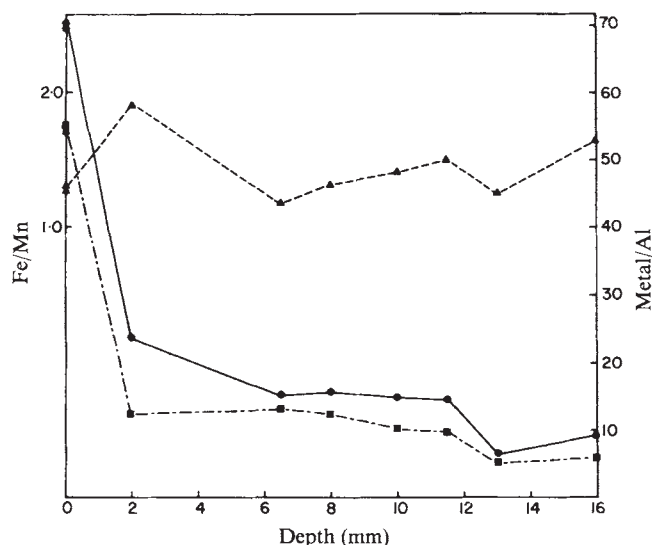


Fig. 2 Fe/Mn (Δ), Fe/Al ($\bullet$) and Mn/Al ($\blacksquare$) ratios as a function of depth in ferromanganese crust as determined by microprobe analyses.

Rise. The minor metals reported in Table 1 for our Fe-Mn crust are all within the reported ranges for these metals in manganese nodules. The concentrations of Ni, Co and Cu, however, are near the minimum values found in nodules and cannot be described as typical. The only mineral detected in the crustal portion of our sample by X-ray diffraction procedures was δ -MnO₂.

Scott *et al.*² suggested that the extreme fractionation of Mn from Fe in deposits from the TAG area may result from the different solubilities of oxides of iron compared with Mn during oxidation by dissolved oxygen¹¹. The reasoning is that Mn tends to be transported further from the source whereas Fe is precipitated at lower levels as oxides or sulphides. If this type of situation is representative of metal transfer in hydrothermal areas of the sea floor, then we may have, by chance, sampled an intermediate zone where both Fe and Mn are being deposited simultaneously. Scott *et al.*⁵ have shown that the Mn-rich hydrothermal deposits from the TAG area are later covered by a more Fe-rich material, possibly of hydrogenous (seawater) origin. Moore and Vogt⁶ have shown the same relation for a sample located near the Galapagos Spreading Center.

Figure 2 presents compositional data obtained at different levels within a polished section of 1052 using an electron microprobe. The Fe/Mn ratio remains between 1 and 2 throughout, but both the Fe/Al and Mn/Al ratios have a steep gradient in the outer portion of the crust. These metal/Al ratios reach levels on the exterior similar to those reported for EPR crest metalliferous sediments¹². The reason for these steep gradients is not clear, although the concentration of volcanic debris (especially laths of plagioclase) was higher toward the interior of the crust.

Like the Fe/Mn ratios, the concentration of rare earth elements (REE) is uniform. The Ce/Fe and Eu/Fe ratios vary by less than 25% for seven of the layers analysed. The curve for the average REE concentrations, normalised to shale, is similar in pattern to that of seawater and crustal sediment from the East Pacific Rise (Fig. 3). This pattern is characterised by a negative Ce anomaly and enrichment of heavy REE relative to light REE. The total concentration of REE, however, is quite high and is in the range of concentrations for ferromanganese nodules, representative of a slowly accumulating manganese deposit. The shape of the curve, however, differs markedly from that of nodules. The curve for Pacific Ocean nodules which give similar X-ray diffraction patterns, usually have a positive Ce anomaly and slight depletion of the heavy (REE) Yb and Lu.

Growth rates

To assess the rate of growth of this deposit, uranium-series isotopes were measured in six contiguous layers throughout the crust. Separation, purification and counting of U and Th isotopes involved standard techniques¹³. The individual sections of the crust were removed by scraping the material from a measured area, weighing the material recovered, and calculating the depth based on an assumed density of 1.96 g cm⁻³ (ref. 14). Because of the configuration of this particular sample (Fig. 1), it was possible to measure directly the thickness of each interval by measuring the remaining thickness after each section was scraped away. The agreement between those two estimates was quite good, and it is estimated that the accuracy of the depth intervals is better than $\pm 10\%$.

When the uranium-unsupported ²³⁰Th activity is plotted relative to depth within the crust, a steep exponential curve results (Fig. 4a). If a constant rate of deposition of the ferromanganese material and a constant flux of ²³⁰Th are assumed, and if the decline of the excess ²³⁰Th is due solely to radioactive decay, we can estimate the rate of accretion of this material by dividing the depth interval over which half the activity of ²³⁰Th disappears by the half life of ²³⁰Th (75,200 yr). The resulting rate, 47.9 mm per 10⁶ yr, is about one order of magnitude higher than typical rates deduced for deep-sea manganese nodules¹⁵. Although this rate is relatively high, there are several lines of evidence which indicate that, in fact, it must have grown considerably faster.

The assumption that the ²³⁰Th gradient is due solely to radioactive decay is suspect because of the strong ²³²Th gradient measured in the outer 8 mm of the crust (Fig. 4a). The slope of the ²³²Th activity in this portion of the crust is so similar to that of ²³⁰Th that when the excess ²³⁰Th is normalised to ²³²Th, there is virtually no slope (Fig. 4b). The deposit would have had to accrete at a minimum rate of about 100 mm per 10⁶ yr to obscure a gradient in the ²³⁰Th/²³²Th ratio.

Another inconsistency in the uranium-series data concerns the isotopic composition of uranium throughout the thickness of the crust. The ²³⁴U/²³⁸U activity ratio for five of the six intervals analysed (Fig. 4b) is within analytical error of that in modern-day ocean water¹⁶. If this crust had accreted at an

Fig. 3 Rare earth elements (REE) in crustal portion of 1052 ($\circ$) and other marine phases normalised to shale. The curve for 'ferromanganese nodule' ($\bullet$) is an analysis²³ of a nodule with similar mineralogy (δ -MnO₂) as the crust from station 1052. The ridge basalt (Δ) analysis is from ref. 24, the East Pacific Rise ($\star$) sediment from ref. 25, and seawater ($\blacksquare$; data $\times 10^6$) from ref. 26.

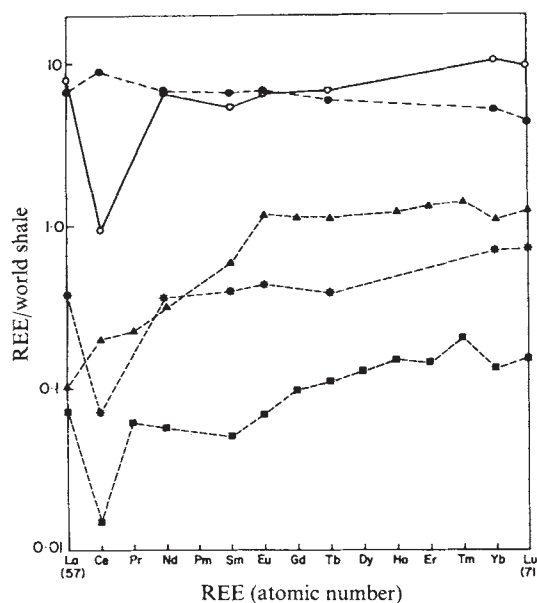
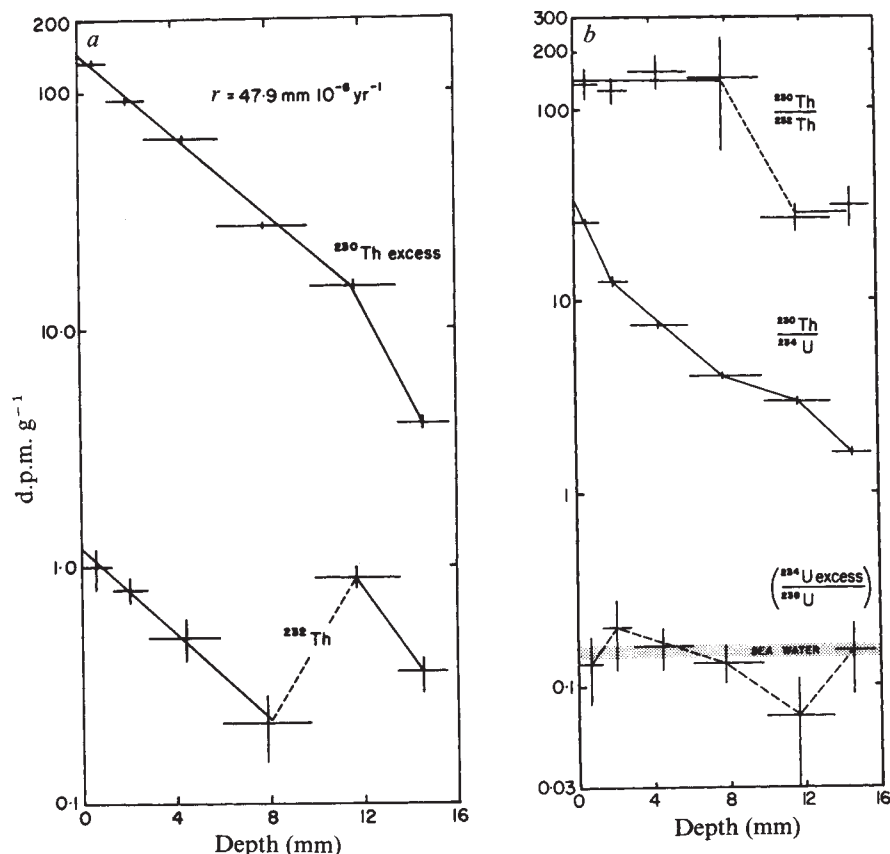


Fig. 4 *a*, Uranium-un-supported ^{230}Th activity and activity of ^{232}Th plotted against depth in ferromanganese layers of 1052. *b*, Activity ratios of $^{230}\text{Th}/^{232}\text{Th}$, $^{230}\text{Th}/^{234}\text{U}$ and ^{234}U excess/ ^{238}U as a function of depth of ferromanganese in sample 1052. Horizontal error bars indicate range in thickness of layer sampled. Vertical error bars indicate 1σ errors as calculated from counting statistics.



average rate of 47.9 mm per 10^6 yr, the age of the base of the crust would be approximately 320,000 yr. If the uranium in the deposit had been derived from seawater and remained a closed system throughout its history, we would expect a $^{234}\text{U}/^{238}\text{U}$ activity ratio of about 1.06 for the basal layer of the crust. This is clearly not the case, and requires that we either had open exchange of uranium between the ferromanganese and seawater or the deposit is quite youthful.

Although it is difficult to reconcile the radiochemical data in terms of a slow rate of growth, we cannot conclusively demonstrate that the deposit formed at a faster or slower rate than calculated here, without an independent assessment of the age of the deposit. Because a well-developed zone of palagonite was present between the fresh basaltic glass and the ferromanganese, we tried to date the glass by hydration-rind techniques. This method, known to archaeologists as 'obsidian dating'¹⁷, has also been applied to geological problems¹⁸. Because of the relative compositional uniformity of submarine basaltic glasses and the very constant ambient temperatures of oceanic bottom waters, the dating of submarine glasses by this technique seems to be reliable^{19,20}. The principal assumption of the hydration-rind method is that the rate of submarine palagonisation of basaltic glass is constant, making it possible to obtain an age of the glass by simply measuring the extent of the hydration. An average rate of submarine palagonisation of $2.91 \mu\text{m}$ per 10^3 yr was obtained²⁰ by measuring the thickness of the hydration rind in submarine samples of known age. This rate has recently been confirmed on samples from the Mid-Atlantic Ridge, in which age was assessed by fission-track dating²¹.

Using a thin section cut normal to the plane of growth of the ferromanganese material, several dozen traverses were made across the hydration rind using a microscope with a calibrated micrometer. This rind can be seen in the photomicrograph (Fig. 1b). The average thickness of the palagonite was $56 \pm 4 \mu\text{m}$. This corresponds to a hydration age of $19,000 \pm 1,500$ yr, much lower than the calculated minimum age of 320,000 yr, for the volcanic glass found by extrapolating the excess ^{230}Th data to the base of the crust.

Another way of estimating the age of this deposit from

radiochemical data is to calculate an excess ^{230}Th 'exposure age'¹⁴. Assuming that the deposit contained no initial excess ^{230}Th but acquired its present-day content by exposure to normal ocean water, we can calculate the duration of this exposure by simply dividing the integrated content of excess ^{230}Th by its flux to the sea floor. The ferromanganese crust contains $145 \text{ d.p.m. } ^{230}\text{Th}_{\text{ex}} \text{ cm}^{-2}$ and the flux of ^{230}Th is calculated to be $2.35 \text{ d.p.m. cm}^{-2}$ per 10^3 yr per km of seawater on the basis of the normal uranium content of seawater with a 15% excess of ^{234}U and assuming quantitative removal of ^{230}Th produced from uranium. The 'exposure age' resulting from this calculating is 18,500 yr, in excellent agreement with the 'hydration age' of 19,000 yr.

Although our exposure age calculation produces an 'age' in apparent agreement with our hydration date, the cause of the exponential gradients of the Th isotopes must still be explained. It seems unlikely that ^{230}Th could diffuse over 15 mm in only 19,000 yr. Scott *et al.*⁵ discussed the apparent inverse relationship between rates of manganese crust accumulation and content of trace metals. If the amount of thorium incorporated into a ferromanganese crust is also inversely proportional to its growth rate, then the gradients that we have measured could simply be a result of the accretion rate slowing down.

Our age estimates for this deposit (1052) are summarised below.

- (1) Extrapolation of rate derived from excess ^{230}Th gradient:
 Apparent rate of growth = 47.9 mm per 10^6 yr
 Thickness of crust = 15.5 mm
 Minimum age of glass = 320,000 yr
- (2) Hydration-rind dating of basaltic glass:
 Rate of submarine palagonitisation = $2.91 \mu\text{m}$ per 10^3 yr
 Average thickness of palagonite rind = $56 \mu\text{m}$
 'Hydration' age of glass = $19,000 \pm 1,500$ yr
- (3) Time required to acquire integrated amount of excess ^{230}Th

$$\sum ^{230}\text{Th}_{\text{ex}} = \int_0^\infty ^{230}\text{Th}_0 \rho \exp(-\lambda^D) dD$$

where $^{230}\text{Th}_0$ = extrapolated surface activity of excess ^{230}Th (d.p.m. g^{-1}); ρ = bulk density (g cm^{-3}); D = depth in crust; $\lambda = 0.693/D^1$ (D^1 = depth at which activity of excess ^{230}Th equals $\frac{1}{2}$ that of surface value) and ^{230}Th flux = 2.35 d.p.m. cm^{-2} per 10^3 yr per km seawater. Depth of crust = 3,340 m.

For 1,052: $\Sigma^{230}\text{Th}_{\text{ex}} = 145$ d.p.m. cm^{-2} . Therefore, 'exposure' age = 18,500 yr.

Based on the correspondence between the ages derived from hydration-rind and ^{230}Th flux techniques, and the fact that the ^{232}Th distribution in the ferromanganese crust precludes radioactive decay being the only process affecting the ^{230}Th gradient, the lower age estimate for this sample would seem to be the more accurate estimate. If the age of the volcanic glass is actually about 19,000 yr, the minimum rate of accretion of the Fe-Mn oxides would be about 800 mm per 10^6 yr. In fact, it must have been even faster, since we have calculated that more than 18,000 yr of exposure to seawater would be necessary for accumulation of the excess ^{230}Th that is present in the crust today. Volcanism could have supplied some of the ionium, but this is unlikely since other hydrothermal deposits sampled from oceanic ridges have been shown to contain no excess ^{230}Th (refs 2 and 6).

Conclusions

Our results show that in our sample from the Hess Deep, first, a ferromanganese crust has been deposited without severe fractionation between Fe and Mn. Second, the trace metals Ni, Co and Cu are low compared with typical manganese nodules but the content of REE is about the same. Third, the REE pattern, normalised to shale, is very similar to the seawater pattern. Fourth, the major assumptions in applying U-series dating techniques for assessing rates of growth seem to fail in this case and we conclude that the deposit must have accreted considerably faster than that indicated by the excess ^{230}Th method.

On the basis of these results, we suggest that Fe and Mn as well as some of the trace metals in this crust were deposited from hydrothermal fluids while REE were scavenged from seawater.

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Electrophoresis along cell membranes

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Bioelectric fields may segregate charged components floating in the plasma membranes of cells by a process of electrophoresis along the membrane. Molecules in cell membranes may be sorted to different portions of the cell surface by such electrical gradients. We present here a theory to support this hypothesis.

MANY molecules and particles are freer to move along the outer membrane of cells than out of this membrane^{1,2}. Many of the externally protruding components bear a high negative charge density³⁻⁶; whereas lecithin (and sphingomyelin) have a substantial positive charge at the calcium ion concentrations typical of extracellular fluids^{7,8}, and neutral lipids such as cholesterol and certain glycolipids are also common in the outer leaflets of cell membranes^{9,10}. If lateral mobility and charge heterogeneity among these membrane components are sufficient, application of a steady field to a cell should substantially redistribute them within the plane of the plasma membrane (Fig. 1). This process may be called surface, lateral, or perhaps perimembrane electrophoresis. I present here a study of the basis and implications of this idea.

Equilibrium electrophoresis along the membrane of a spherical cell

A complete theory of lateral electrophoresis is analogous to the theory of sedimentation. Both the equilibrium with back diffusion and the approach to this equilibrium must be considered. For the present purpose, only the simpler and apparently more useful equilibrium theory will be presented.

Consider a spherical cell which bears a population of one species of negatively charged molecules or particles on its surface, and which is placed in a uniform field (Fig. 1). Neglect any interactions (other than hydrodynamic drag) between various surface components. Allow them to come to equilibrium between electrophoresis which tends to concentrate them at the positive pole and back diffusion. It will be shown that the equilibrium surface concentration, C , is given by

$$C = \bar{C}[\varepsilon \cdot \text{csch}(\varepsilon)]e^{\varepsilon \cos \theta}, \quad (1)$$

where $\bar{C}$ = the particles' average concentration, $\varepsilon = (m/D) \cdot (\bar{V}/2)$, m = electrophoretic mobility, D = diffusion constant,

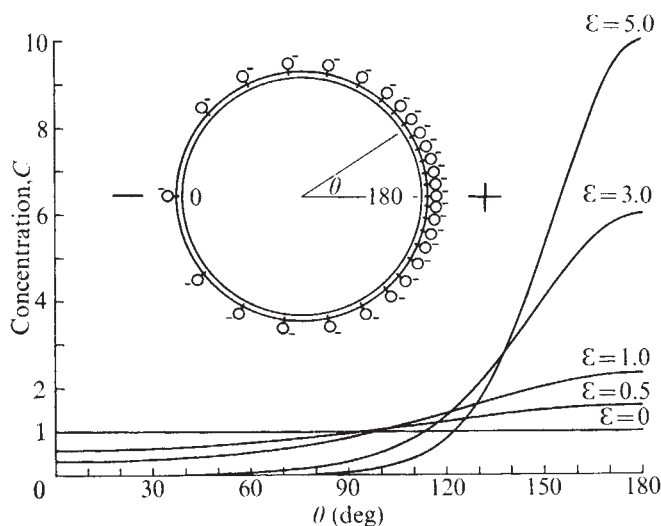


Fig. 1 Distributions of mobile charged particles on the surface of a sphere at electrophoretic equilibrium. Curves plot equation (1).

and $\bar{V}$ = voltage difference established across the sphere by the field. This is plotted in Fig. 1.

The particles are at a true equilibrium between several forces produced by the external field and Brownian movement, so it might be supposed that a Boltzmann distribution could be applied. Their counter ions are not, however, in equilibrium but in a steady state, so this most direct method cannot be used. Instead one writes the equation for a balance between electrophoretic and diffusional transport

$$mC(dV/dS) = D(dC/dS) \quad (2)$$

A sphere is known to distort an otherwise uniform field so that the potential just outside its surface, V , remains proportional to $\cos\theta$ (ref. 11). Hence

$$V = (\bar{V}/2) \cos\theta \quad (3)$$

Substituting equation (3) in (2) and integrating yields equation (1).

Exactly the same derivation holds for the distribution of particles attached to the inner surface of a cell's membrane and concentrated by a uniform internal field, except that in this internal case $\bar{V}$ would refer to the transcytoplasmic potential difference. A uniform internal field might be set up by either a uniform external one¹¹ or by a cosine varying current source in the cell's own plasma membrane¹².

For comparison with cells' galvanotropic responses (or perhaps other polar responses) it is helpful to characterise the degree of polarisation of each of these equilibrium distributions¹ by some parameter, ϕ . The tropistic responses of cell populations are commonly characterised by the weighted cosine of the distribution of outgrowth angles (13)

$$V_1 = \Sigma n \cos\theta / \Sigma n \quad (4)$$

where n is the number of outgrowths at an angle, θ .

Hence we define ϕ in an analogous way

$$\phi = \int C \cos\theta \, ds / \int C \, dS \quad (5)$$

Note that if the outgrowth angles or surface particles were all concentrated at one pole or the other, V_1 and ϕ would be $+1$ or -1 whereas if they were evenly distributed, these parameters would have a value of zero.

Integration of equation (5) yields

$$\phi = \text{ctnh}(\epsilon) - \epsilon^{-1} \quad (6)$$

Use of Pierce's series¹⁴ number 793 yields

$$\phi = \epsilon/3 - 0.133 \epsilon^3 + 0.127 \epsilon^5 \dots \quad (6a)$$

This suggests that for $\epsilon < 1$, ϕ is usefully approximated by

$$\phi \simeq \epsilon/3 = (m/D)(\bar{V}/6). \quad (6b)$$

Equations (6) and (6b) are plotted in Fig. 2.

Expected polarisability of real membrane particle distributions

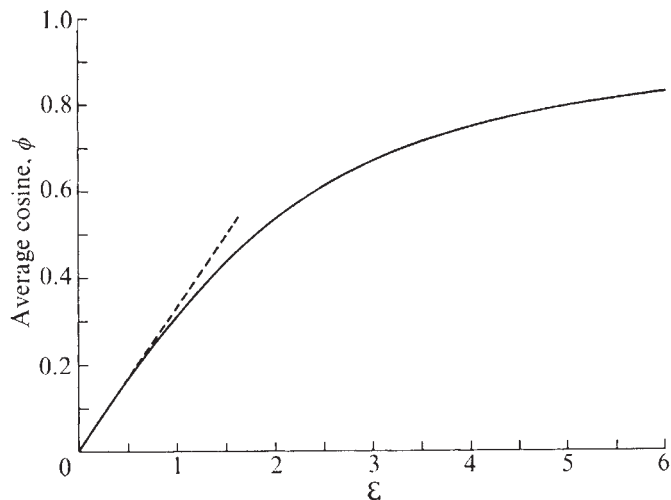
Let us characterise the polarisability of a particle distribution on a cell by the voltage $\bar{V}_{0.1}$ required to induce a polarisation of 0.1. From equation (6b) one gets

$$V_{0.1} = 0.6 (D/m) \quad (6c)$$

To estimate this voltage, one must estimate the diffusion to mobility ratio, D/m of the particles being redistributed. We will show below that the larger and more highly charged particles found in cell membranes are likely to have D/m values of about a millivolt. So we predict that long-lasting fields, whether endogenous or imposed, are likely to bring about significant electrophoresis along cell membranes when the voltage drop per cell is only one to a few millivolts.

To estimate D/m values, we will first show that D/m is independent of the drag, f , which they encounter. This drag is an estimated 100–1,000 times greater than such particles would meet if they were only hindered by the extracellular medium². If this greater drag were exerted by the viscosity of the medium which fills and surrounds their electrical double layer, then D and m are both well known to vary with f^{-1} . Hence D/m would be independent of f . It is likely, however, that this extra drag is exerted on a sort of molecular clog, that is, a part or extension of each particle that lies within the lipid bilayer and is not surrounded by a field-sensitive double layer; whereas the electrical pull is exerted on each particle's 'sail', that is, its part that extends outside of the bilayer in the external aqueous phase (Fig. 1). Then m would equal F , the net electrophoretic force per field (which acts on the sail), divided by the drag which acts on the clog. Henry's analysis¹⁵, directly confirmed by Sumner and Henry's experiment¹⁶, shows F to be unchanged whether the particle is free or tethered. Hence m is inversely proportional to the drag wherever it originates. Since this also holds for D (ref. 17), D/m is again independent of the drag.

Fig. 2 Average cosines of the distributions shown in Fig. 1. The curve plots equation (6); the dashed line, equation (6b).



Since D/m values are independent of the drag, we can use the values known for molecules or particles immersed in the usual 1-cP aqueous media. In media with this viscosity (and an ionic strength of 0.05 M) most biological molecules or particles have electrophoretic mobilities in the range from 1 to 3, or occasionally up to $5 \times 10^{-4} \text{ cm s}^{-1} (\text{V cm}^{-1})^{-1}$ (refs 5, 18). Electrophoretic mobilities are independent of the component's radius, but diffusion constants are inversely proportional to it. So to estimate how small a voltage drop per cell may be expected to bring about significant redistribution, let us consider relatively large membrane particles. Let us consider spherical particles with 10-nm diameters since the larger particles seen in freeze-fracture electron micrographs of plasma membranes are of about that size and shape¹⁹⁻²³. In 1-cP media, such 10-nm spheres have a diffusion constant of $4 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ (ref. 24). Then considering particles with a mobility value of 3×10^{-4} units, which is towards the upper end of those usually found, one obtains a D/m value of 1.3 mV and applying equation (6) we infer that the distribution of such particles would be one tenth to one half polarised by a steady voltage drop of only 0.8–4.0 mV.

Application of this equilibrium theory is restricted to the action of sufficiently long-lasting fields. The time needed is of the order of L^2/D where L is the cell's pertinent diameter and D the segregated components diffusion constant. The larger mobile components of cell membranes tend to have diffusion constants in the vicinity of $10^{-9} \text{ cm}^2 \text{ s}^{-1}$ (ref. 2), so for a 30- μm (that is, $3 \times 10^{-3} \text{ cm}$) cell, the equilibrium approach time might be about 10^4 s , or 3 h. When one considers that the bioelectric fields associated with growth and development generally last for hours to days or even weeks (that is, 10^4 to 10^6 s), one concludes that an equilibrium theory may be widely relevant.

The accompanying paper, by Poo and Robinson²⁵ seems to directly demonstrate the predicted phenomenon of lateral electrophoresis along the surface of explanted, embryonic muscle cells exposed to an artificially imposed field. Moreover, a number of galvanotropic responses of isolated plant cells to artificial fields occur in response to such small voltage drops as to suggest mediation by lateral electrophoresis²⁶⁻²⁸.

Natural fields along cell membranes

Perhaps the most obvious place where large endogenous voltage drops occur along cell membranes is along the outside of the lateral membranes of cells in various metazoan epithelia. Various mature epithelia are well known to maintain voltage drops of 30–100 mV or more across themselves²⁹. Furthermore, there are several reports of 3–10 mV potential drops across embryonic cell layers, for example, the mammalian blastocyst wall³⁰, a fish gastrula wall³¹, and the chick chorioallantoic membrane³². A second place where good evidence for significant steady potentials drops along cell membranes can be cited is along the outer plasma membranes of epidermal cells in the coleoptiles of oat seedlings. A voltage drop of at least

40 mV (refs 33, 34) is measured along the axis of these organs and hence across about 30 cells in series³⁵; whereas up to 80 or 90 mV appears across the axis in response to a gravitational stimulus^{36, 37} and thus across about 100 surface cells in series³⁵. Thus, in both directions, about 1 mV per cell is present.

There are, moreover, at least three instances in which there is evidence that cells generate large enough cytoplasmic fields within themselves electrophoretically to segregate components of the inner leaflet of the plasma membrane. Woodruff and Telfer have quite directly measured a voltage drop of about 10 mV across the cytoplasmic bridge joining a developing insect oocyte and its nurse cell³⁸; Hagins has measured a steady dark current outside of rat visual rod cells and infers an internal current sufficient to maintain a steady difference of about 1.6 mV along the interior of the cell³⁹; while Robinson and Jaffe have measured a calcium current through developing fucoid eggs which might maintain the order of a millivolt difference across the interior of the cell^{40, 41}.

I conclude that endogenous voltage gradients sufficient to bring about electrophoresis along cell membranes are very widespread in living organisms.

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Electrophoresis of concanavalin A receptors along embryonic muscle cell membrane

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Fluorescent concanavalin A (con A)-labelling showed that an electric field of 4 V cm^{-1} grossly redistributed con A receptors along the plasma membranes of living muscle cells within 4 h. This field produced a voltage drop of 12 mV across these 30 μm -wide cells. The move-

ment of receptors was independent of cell metabolism and seemed to be electrophoretic in nature.

STUDIES of the cell membrane have demonstrated the dynamic nature of membrane constituents¹⁻⁵. Both proteins and lipids may undergo rotational and translational diffusion in the plane of the membrane⁶. In the accompanying paper⁷, Jaffe demonstrates on theoretical grounds that small voltage

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differences (of the order of 1 mV across a cell) applied parallel to the surface of cells should be effective in redistributing charged, mobile entities in the plane of the membrane. We have placed cultured embryonic amphibian muscle cells in a field of 4 V cm^{-1} and examined the subsequent distribution of the receptors of concanavalin A (con A). We found, using fluorescein isothiocyanate (FITC) labelled con A, that these receptors become asymmetrically arranged within a few hours. The receptors (presumably glycoproteins) accumulated on the side of the cells nearer the negative pole in a way consistent with a passive electrophoretic mechanism.

Muscle cells were obtained from the neural tube region of 1-d-old *Xenopus laevis* embryos (stage 18–19 by the criterion of Nieuwkoop and Faber⁸). After removal of the vitelline membrane, the neural tube and the underlying mesoderm were cut from the embryo in Ca^{2+} -, Mg^{2+} -free Steinberg's solution containing 0.4 mM EDTA (ref. 9). When the cells became dissociated, they were transferred to a shallow, rectangular glass chamber which was pre-coated with poly-L-lysine. The cells were cultured in Steinberg's solution supplemented with 10% Leibovits medium (L15, GIBCO) and 5% foetal calf serum (GIBCO). The pH of the culture medium was 7.8. Each culture contained various cell types from a single embryo and was incubated at room temperature (about 25°C). Although neurones and other cell types survive for a while, after 2 d the predominate cells in the culture were single, spindle-shaped, striated muscle cells. These cells responded to 0.01% carbachol (Sigma) by contracting from both ends into a sphere.

The 0.5-mm deep chamber was covered with a coverslip before application of the electric field, thus providing a defined geometry. The current was applied through a pair

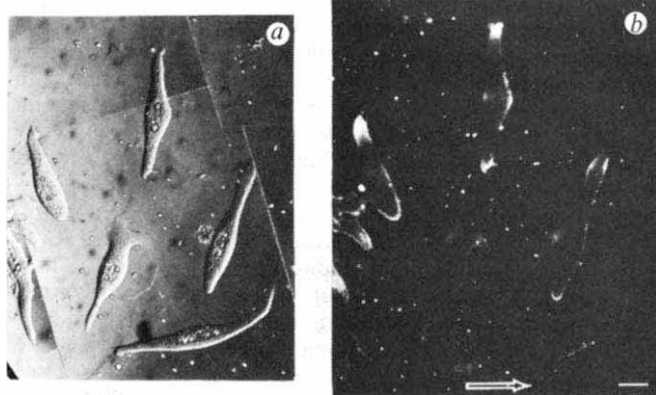


Fig. 1 FITC-con A-labelled living muscle cells observed by *a*, light microscope fitted with Normarski optics and *b*, dark-field fluorescence microscope. Embryonic muscle cells in monolayer culture exposed to a uniform electric field (4 V cm^{-1}) for 4.5 h before staining. Fluorescent 'ring' showed asymmetry for nearly all cells in the culture, with more stain accumulated towards the negative pole of the field (to the right of the figure). The brighter stain near the tips of some cells was due to partial contraction of the cells which we assume caused folding of the membrane resulting in higher concentration of con A receptors. Fluorescent spots in the background and on the surface of some cells were due to stained cell debris. Direct visual comparison of the fluorescence intensity on two sides of the cells were carried out for all cells which were aligned relatively perpendicular to the direction of the field. Labelling was done at $0-5^\circ\text{C}$ for 10 min. Bar: $30 \mu\text{m}$.

aldehyde fixation caused marked autofluorescence in these muscle cells, and glutaraldehyde thus was an unsuitable fixative.

The fluorescent microscopy was done using a standard Zeiss microscope fitted with a dark-field condenser. The

Table 1 Con A receptor distribution in electrical field

Duration of electric field application* (and voltage drop across each cell)	No. of cells examined†	No. of cells showing asymmetry in the fluorescence 'ring' staining			Description of the asymmetry in fluorescence
		Towards (–) pole	Towards (+) pole	Indeterminant	
0	53	7 (13%)	10 (19%)	36	no detectable asymmetry
30 min (12 mV)	77	18 (23%)	16 (21%)	43	no detectable asymmetry
1.5 h (12 mV)	103	67 (65%)	11 (11%)	25	weak but definite asymmetry
4.5 h (12 mV)	86	70 (81%)	3 (3%)	13	striking asymmetry
14 h (12 mV)	27	25 (92%)	0 (0%)	2	striking asymmetry
24 h (0.6 mV)	60	27 (45%)	9 (15%)	24	weak but definite asymmetry

*All data for 12 mV voltage drop are from the same experiment; data for 0.6 mV voltage drop are from two other experiments.

†Cells were randomly sampled with only one criterion: their long axes were aligned within 30° degrees with respect to the direction normal to the field direction, so that the fluorescence staining on two sides of the cell could be compared (see text for detail).

of 8-cm-long bridges filled with Steinberg's solution gelled with 2% agar. These agar bridges isolated the cultures from contamination by the Ag–AgCl electrodes, to which a regulated power supply was connected. The applied fields were calculated from the current (1.5 mA usually), the medium's conductivity ($130 \Omega \text{ cm}$), and the chamber's cross section (0.05 cm^2). The usual current of 1.5 mA produced a field of 4 V cm^{-1} in the chamber.

After the field was shut off, the cells were cooled to $0-5^\circ\text{C}$, the culture medium was washed off with Steinberg's solution, and then Steinberg's solution containing $200 \mu\text{g ml}^{-1}$ FITC-con A (Miles) was introduced into the chamber. After 10 min, the FITC-con A was removed with Steinberg's solution. In some cases the cells were then fixed in cold acetone (-5 to 0°C) and preserved in 90% glycerol; in other cases, the living cells were examined by conventional and fluorescent microscopy. We found that glutar-

excitation source was a Zeiss HBO 200W/4 lamp. Exciting light was passed through a heat-absorbing filter and a BG 12 blue filter, and emitted light was filtered with a No. 50 barrier filter.

Redistribution of con A receptors

In control cultures, where no electric field was applied, the FITC-con A-labelled cells showed uniform staining with a bright ring around the cell periphery since the surface is seen edge-on there (Fig. 2*a*). This ring was our most reliable and sensitive indicator of receptor redistribution. The labelling was specific, since it was completely blocked by 0.1 M α -methyl-D-mannoside, one of the sugars for which con A is specific¹⁰. Further, most of the label on prelabelled cells was removed by applying 0.1 M α -methyl-D-mannoside for 10 min.

When an electric field of 4 V cm^{-1} was applied to cell cultures for 4.5 h, nearly all cells whose long axis was perpendicular to the field showed a striking asymmetry in their ring staining pattern, with more label appearing on the side of the cells facing the negative electrode (Fig. 1b). Bright-field microscopic observations (both phase-contrast and Nomarski) were made on the same cells and no morphological difference corresponding to the observed asymmetry in fluorescence staining could be seen (Fig. 1a). Cells that were oriented with their long axes parallel to the field also showed some accumulation of label at the negative-facing tip, but this effect was much smaller and harder to score than the asymmetry along the short axis.

experiment; similar results were obtained in four separate experiments. We concluded that the con A receptor moves halfway across the surface of the $30\text{-}\mu\text{m}$ -wide muscle cell in 1–2 h.

After a field as small as 0.2 V cm^{-1} (corresponding to a potential difference of 0.6 mV across a $30\text{-}\mu\text{m}$ cell) was applied to a culture for 24 h, a detectable asymmetry in the con A receptors was found (see Table 1). Fields smaller than 0.2 mV cm^{-1} did not cause detectable redistribution.

We have considered the possibility that the apparent positive charge of the con A receptors was due to the polycation, poly-L-lysine, that was used to coat the surface of the glass on which the cells were grown. The cells grew

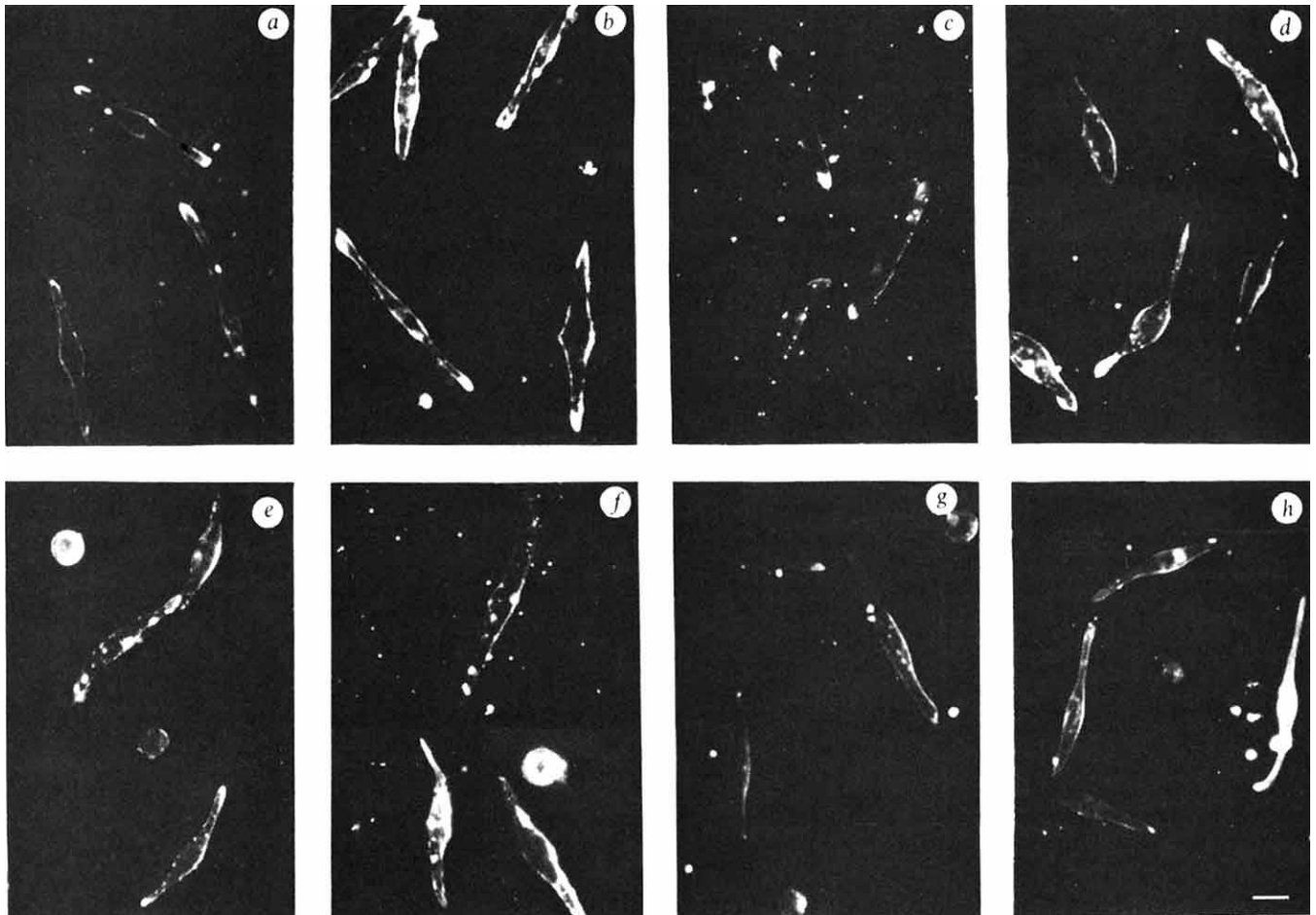


Fig. 2 *a*, Control culture, where no field has been applied, FITC-con A-labelled cells showed uniform staining with a bright ring around the cell periphery. *b*, *c*, *d*, Cells were exposed to an electric field of 4 V cm^{-1} for 30 min, 1.5 h, and 4.5 h, respectively, before FITC-con A labelling. *e*, Cells were preincubated in Steinberg's solution containing 10^{-2} M sodium azide and 10^{-3} M dinitrophenol for 2 h. A field of 4 V cm^{-1} was then applied for 2 h before labelling. Both field application and FITC-con A labelling were carried out in the presence of these compounds. *f*, Cells treated as in (*e*) except the Steinberg's solution contained 10^{-2} M sodium arsenate, 10^{-3} M sodium fluoride, and 10^{-3} M dinitrophenol. *g*, Cells treated as those in (*d*) except Ca^{2+} was omitted from the medium during the application of the field. *h*, Cells treated as those in (*d*) except FITC-conjugated wheat germ agglutinin was used as a fluorescent label instead of FITC-con A. All cells were fixed with cold acetone (at 0 to $+5^\circ \text{C}$) after labelling. Negative pole of the field was on the right of the figure. Bar: $30 \mu\text{m}$.

To investigate the time course of the redistribution, we applied the same field (4 V cm^{-1}) to a group of cultures for 30 min, 1.5 h, 4.5 h, and 14 h. The asymmetry in fluorescence staining on cells which had their long axes approximately perpendicular to the field was then scored (see Fig. 2*a–d*). We found that the effect was clearly perceivable after 1.5 h in the field and that it did not seem to increase beyond 4.5 h. Table 1 shows data from one

well on clean, untreated glass, so the experiment was repeated in the absence of poly-L-lysine, with a field of 4 V cm^{-1} . The same obvious accumulation of the con A receptors on the negative side of the cell was seen after 4 h.

In two separate experiments, FITC-wheat germ agglutinin (WGA) was added to cells which were in a 4 V cm^{-1} field for 4.5 h. Some cells seemed to show a slight accumulation towards the negative side (Fig. 2*h*); however, a count of cells

such as was done for Table 1 showed that the asymmetry was less than was seen for the con A receptors after 1 h in the field. The WGA receptor is thus either much less charged or much less mobile than the con A receptor.

Mechanism of receptor redistribution

We considered the redistribution of the con A receptors in an electric field to be a passive, electrophoretic phenomenon. As an alternative, however, the field might somehow align and activate a motive system in the cortex of the cell which then actively redistributes surface components, including the con A receptors. Since any such system would presumably require metabolic energy, we performed experiments in the presence of metabolic inhibitors. First, we preincubated the cells in Steinberg's solution containing both 10^{-2} M sodium azide and 10^{-3} M dinitrophenol, a treatment known to prevent con A-induced capping in other cells^{11,12}. As a second, more drastic test, we preincubated other cells in a medium including 10^{-2} M sodium arsenate, 10^{-3} M sodium fluoride, and 10^{-3} M dinitrophenol for 45 min. This latter treatment blocked their response to 0.01% carbachol, a carbachol concentration which caused instantaneous contraction in control cultures. This indicated that the cellular energy supply for the contractile machinery had been exhausted. Cells so pretreated were then placed in an electric field (still in the presence of the respective metabolic inhibitors) and the distribution of the con A receptors examined as before. Neither treatment had any effect: the con A receptors still accumulated on the negative side with the same time course and to the same extent as in control cells (see Fig. 2e, 2f). Similarly, the presence of 5×10^{-6} M colchicine throughout the experiment had no effect on the redistribution. Moreover, when Ca^{2+} was omitted from the medium ($[\text{Ca}^{2+}] < 10^{-5}$ M) during the application of the field, the redistribution proceeded as in the normal Ca^{2+} medium (Fig. 2g).

We therefore believe that the observed redistribution of the con A receptors is a passive response of these molecules to the electric field. As will be discussed later, the estimate of receptor mobility from these data is consistent with the idea of electrophoretic movement of receptor molecules through an environment with a drag similar to the measured drag on other cell membrane constituents⁶. Moreover, if the redistribution of the con A receptors is a passive electrophoretic movement, removal of the electric field after the receptor is asymmetrically distributed should allow a relaxation of the asymmetry due to back diffusion. Preliminary results indicated that if cells were allowed to remain in the culture medium following the application of a field but before the application of FITC-con A, there was a partial relaxation of the asymmetry. Some recovery to the original uniform distribution was apparent after 3 h. About 50% of the cells examined, however, still showed a weak asymmetry in the receptor distribution even after 54 h. Similar results were obtained in three separate experiments. The characteristic $1/e$ time for the partial relaxation of asymmetry is about 10 h (range 5–15 h). Relaxation experiments have not been performed in the conditions inhibiting metabolism, since the cells die if treated with drugs for the longer times required in these experiments.

Discussion

An externally applied electric field may exert its action on cultured muscle cells in several ways. It may act by producing a cytoplasmic potential gradient. It may act by changing the membrane potential on the two sides of the cell. It may also act tangentially along the surface of the cell. In our experiments, the potential difference produced by the field across a perpendicularly aligned muscle cell (average width $30 \mu\text{m}$) was about 12 mV. Since only a very small fraction

of the external voltage drop would lie across the cytoplasm, the directed movement of cytoplasmic components which could in turn redistribute surface receptors seems quite unlikely. The membrane potential on the two sides of the cell facing the electrodes will be shifted by about 6 mV. It is not clear, however, how this steady shift in membrane potential could have caused the observed redistribution of the receptor molecules in the plane of the membrane. Rather, we propose that the primary action of the applied field is to act tangentially along the membrane^{7,13}. An external tangential field would be experienced by the charged, protruding heads of membrane-bound molecules, and these molecules would thus be driven (to the extent that they are mobile) towards the anodal or cathodal side of the cell. Quantitatively, this mechanism is a plausible explanation of the redistribution of con A receptors which we have observed (see ref. 7).

A crude estimate of receptor mobility can be made from the data on the time course of the redistribution. We take 2 h as the time needed for a receptor molecule to move approximately half way across the width of the cell, that is, the average electrophoretic mobility of the receptor is about $15 \mu\text{m}$ per 2 h per 4 (V cm^{-1}), or about $5 \times 10^{-4} \mu\text{m s}^{-1}$ (V cm^{-1})⁻¹, a value 2×10^3 fold smaller than the electrophoretic mobility of a typical globular protein in an aqueous medium. The viscous drag experienced by the receptor molecules undergoing electrophoretic movement in the plane of the membrane is thus similar to that imposed by a medium having a viscosity of about 20 P (since the viscosity of water is 1 cP), which is close to but higher than other estimates of membrane viscosity⁶. Experiments on the relaxation of the asymmetry after the field is turned off suggest a diffusion coefficient for lateral diffusion of the mobile unlabelled receptor of $3 \times 10^{-11} \text{cm}^2 \text{s}^{-1}$ (range $2\text{--}6 \times 10^{-11} \text{cm}^2 \text{s}^{-1}$), since the diffusion coefficient, $D = r^2/2t$, where r is the radius of the cell ($\sim 15 \mu\text{m}$), and t is the $1/e$ relaxation time¹⁴. This is a value comparable to those reported for mobile membrane proteins diffusing in the plane of the membrane^{5,6,15–18}.

The accumulation of con A receptors on the negative side of these muscle cells indicates that the majority of these molecules are either positively charged or else less negatively charged than other displaceable membrane components. This agrees with the finding of Skutelsky and Farquhar that the con A receptors of developing red blood cells have relatively little affinity for positively charged colloidal ion particles⁹.

Finally, we expect that the *in situ* electrophoresis of cell surface components demonstrated in this report will prove to be a useful technique for measuring receptor mobility in the plane of the membrane and for exploring the molecular properties of cell-surface receptors within their natural membrane environment.

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letters to nature

Recurrent X-ray outbursts from Aquila X-1

A HIGH degree of variability has been associated with Aquila X-1 (Aql X-1) (3U1908+00) since its occasional observation in early rocket surveys¹⁻³, and its appearance as a variable source of moderate brightness in the Uhuru catalogue⁴ ($S \sim 0.07-0.2 S_{\text{crab}}$). Later observations of the source have been made from OSO-7 (ref. 5) during 1971-73 which suggest the existence of 'low' ($S \sim 0.02 S_{\text{crab}}$) and 'high' ($S \sim 0.5 S_{\text{crab}}$) intensity states, and from OAO Copernicus, where the source was reported at levels at least an order of magnitude below the Uhuru range during intermittent exposures over a 2-yr period through 1975 May (ref. 6). This apparent quiescent state was interrupted in 1975 June by a sudden flare⁷ (factor of >20 increase) to the level of the Crab nebula. Observations of the declining source by the Sky Survey Instrument (SSI) on Ariel V

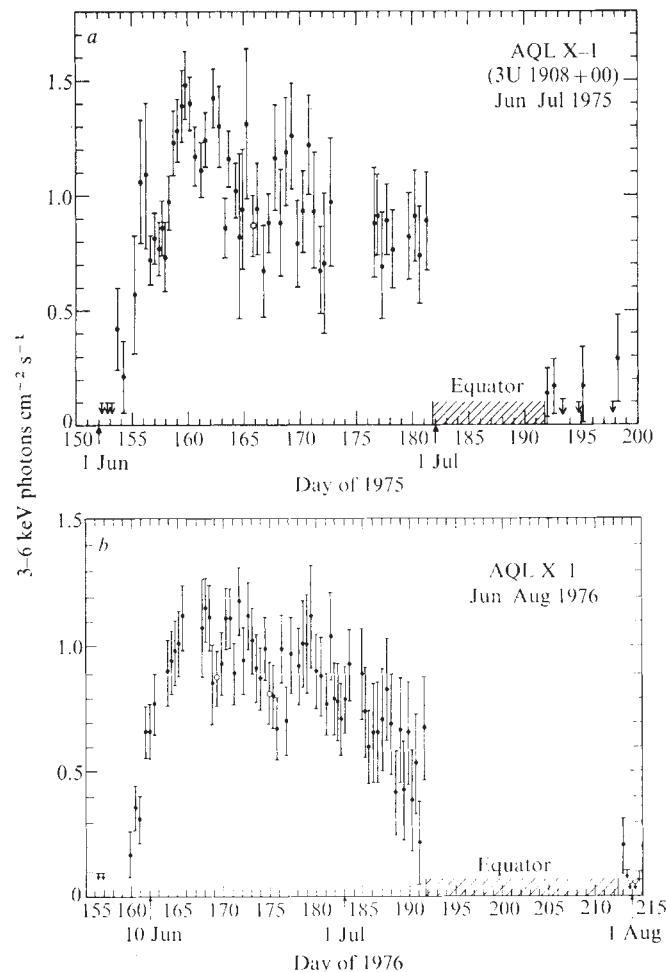
revealed an $\sim 10\%$ sinusoidal modulation⁸ (2-18 keV) at the period $P = 1.3 \pm 0.04$ d. By August the flux had decayed back to the pre-outburst intensity ($< 0.003 S_{\text{crab}}$) as measured by the SSI (ref. 9), which instrument observed the same low flux from Aql X-1 again in 1976 January. In 1976 June, the All-Sky Monitor (ASM) on Ariel V detected an additional outburst of this source¹⁰, which was confirmed by the SSI (M. G. Watson, personal communication). We report here ASM observations of Aql X-1 obtained between the launch of Ariel V in 1974 October and 1976 November, and compare them with the previously cited additional data available. We suggest that the totality of these measurements imply that Aql X-1 may provide a plausible connection between dwarf novae and the very bright, long-duration transient X-ray source¹¹.

The proximity of Aql X-1 to the sources Ser X-1 (3U1837+04) and 3U1901+03 ($S_{\text{max}} \sim 0.3 S_{\text{crab}}$ and $0.1 S_{\text{crab}}$, respectively) make unambiguous detection of a low Aql X-1 flux ($\lesssim 0.2 S_{\text{crab}}$) problematic in the ASM standard resolution mode (see ref. 12 for a complete experiment description). Consequently, occasional fluctuations in detector background and other systematic effects could conceivably result in 'accidental' flux measurements (with low probability) as high as $\sim 0.3 S_{\text{crab}}$ for this particular source. The data obtained during the 1975 and 1976 flares of Aql X-1 are illustrated in Fig. 1, and represent the only unambiguous detections of the source over the ~ 25 months of ASM operation during which the duty cycle for daily monitoring of the Aquila-Serpens region was $> 75\%$. These plots consist of $\sim \frac{1}{2}$ -d averages of the 3-6-keV flux (incident photons) with corresponding $\pm 1\sigma$ statistical error bars (estimated 1σ systematic errors are smaller than the statistical uncertainties). The upper limits obtained ($0.1 \text{ cm}^{-2} \text{ s}^{-1}$) immediately before the 1975 flare are consistent with the SAS-3 observations⁷, and no positive detections of the source above $\sim 0.2 \text{ cm}^{-2} \text{ s}^{-1}$ were made during the preceding $7\frac{1}{2}$ months. A similar search of the ASM data over the period between the flares yielded no unambiguous source sightings through 1976 April, with a possible detection at $\sim 0.3 \text{ cm}^{-2} \text{ s}^{-1}$ (for ~ 1 d) 2 weeks before the 1976 outburst. We note finally that the apparently differing peak flux levels of the flares are actually consistent with a single limiting value ($S_{\text{max}} \approx S_{\text{crab}}$), as degradation in the effective 3-6-keV detector efficiency of $\sim 10\%$ between the two flare observations cannot be excluded.

As a 1.3-d modulation has been reported⁸ from the 1975 SSI flare data (obtained during the time interval in Fig. 1a labelled 'equator'), we have investigated both the 1975 and 1976 ASM data for periods in the range 0.3-3 d. The technique used consisted of separately folding the data over the range of trial periods and noting deviations in the χ^2 -period distribution to the hypothesis of source constancy. The most prominent peak in the 1975 ASM data is, indeed, consistent with the SSI result. As the two experiments have mutually exclusive fields-of-view, the ASM result at a level of $3\% \pm 1\%$ is an independent measure of the same effect, but cannot be claimed to be an independent detection owing to its marginal statistical significance. Assuming its reality, we can refine the period estimation to 1.28 ± 0.02 d. No such modulation is evident at this period in the 1976 data, however, with statistical errors comparable to those in 1975.

A summary of the totality of the 1971-76 data is displayed in Fig. 2, from which there is a clear indication that the OSO-7 data are consistent with flaring episodes similar to those in 1975-76. As there is no exact period which can be fitted to the four flares for which there is observational evidence, we have derived

Fig. 1 ASM chronologies of the 1975 (a) and 1976 (b) flares of Aql X-1. The points are $\sim \frac{1}{2}$ -d averages, and the error bars are $\pm 1\sigma$ (statistical error only). The shaded areas labelled 'equator' are times when Aql X-1 was close to the equatorial plane of the Ariel V satellite; during these times the source was inaccessible to the ASM, but observable by the SSI.



a mean flare interval of 435 d with an r.m.s. scatter of $\sim 10\%$, and have indicated a possible history of identical flares which satisfy all of the observations.

The irregularity of the proposed flare cycle has several obvious implications for source models. In particular, Jones *et al.*¹³ have recently reported recurrent flares from 3U1630–47 similar to those discussed here, but which exhibited a regular period of $P = 615 \pm 5$ d. Those authors have suggested that the 3U1630–47 flare cycle may represent the orbital period of an eccentric binary system (as has also been suggested for several transient sources^{14,15}), but the observed scatter in the presumed Aql X-1 flare period is inconsistent with the relatively precise timing expected from binary phase-related variations in the accretion flow. The same consideration lessens the likelihood of an association of the present behaviour with a Mira Variable-type

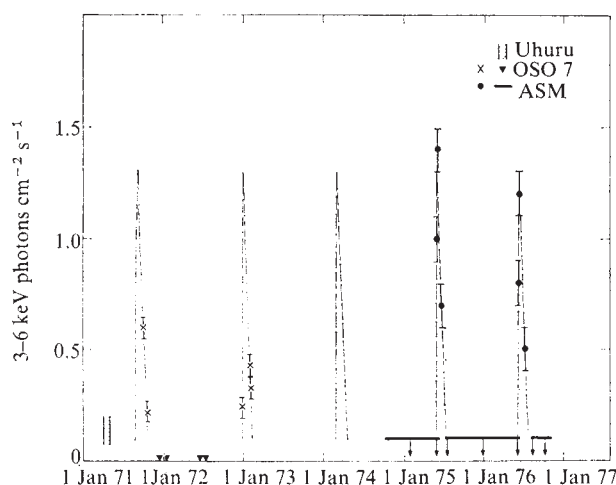


Fig. 2 Summary of Aql X-1 observations over the period 1971–76. The OSO-7 data is taken from Markert⁵ and has been approximately converted to ASM units via normalisation with respect to the Crab nebula. Error bars in all data have been conservatively estimated to include possible systematic and normalisation errors. Intermittent Copernicus⁶ measurements during 1973–75 (not displayed) are at the level ~ 0.01 – 0.02 , and do not preclude a 1974 outburst as hypothesised.

binary as proposed for the transients 3U1543–47 (ref. 16) and A1118–61 (ref. 18), or with a Her X-1-like precession-governed modulation^{18,19} (as opposed to actual flares).

We suggest that the observations are most easily interpreted in terms of an 'X-ray dwarf nova' binary of the type proposed by Avni, Fabian, and Pringle²⁰ for the transient source A0620–00, as the observed X-ray phenomenology is not unlike that of the optical light curves of dwarf novae (and some recurrent novae). Using the empirical period–amplitude relation of Payne-Gaposchkin²¹ for U Gem stars and recurrent novae, the inferred luminosity ratio $L_{\max}/L_{\min} \sim 500$ (assuming that most of the radiation emerges in X rays) yields an expectation value for the interval between flaring episodes of ~ 465 d. The excellent agreement between the predicted and observed mean flare cycles should not be taken literally, as the uncertainty in $L_{\max}/L_{\min}$ implies a correspondingly wide dispersion in the expectation flare interval. Furthermore, X-ray dwarf novae may deviate significantly from period–amplitude relations describing their optical analogues^{20,22}, so we can conclude only that the observed flare interval is not inconsistent with that which might be expected from such a source. The close similarity of the two ASM flare light curves is another model constraint, as it suggests detailed reproducibility. This characteristic of the source behaviour can be reconciled with accretion dwarf nova models

invoking relatively continuous mass transfer to an accretion disk which is eventually 'dumped' by an instability²³ or, alternatively, quasi-periodic unstable Roche-lobe overflow of the red star²². In the former model, the similarity of the flares results from the comparable amounts of material accumulated between episodes, while in the latter a high regularity of the magnitude of Roche-lobe spills and/or a feedback loop (regulated first by self-excited transfer and later by Eddington-limited flow) may be implied. Finally, we note that the non-flaring source behaviour observed by Uhuru (C. Jones, personal communication) may be analogous to the 'standstills' observed in several dwarf novae including Z Cam²³ ($S_{\text{standstill}} \sim 0.3$ – $0.5 S_{\max}$).

The similarity of the Aql X-1 flares reported here to the more extreme episodes of the transient X-ray sources has been pointed out by several observers^{8,24}. The abrupt ($\lesssim 1$ week) rise by ~ 2 order of magnitude and subsequent gradual nova-like decay (e -folding time ~ 1 month) back to the pre-flare level are reminiscent of the characteristic transient source light curves, apparently differing only in the degree of brightening ($L_{\max}/L_{\min}$ for transients is, by definition, $\gtrsim 10^3$) and the absence of a clear precursor peak. This behaviour is, on the other hand, qualitatively different from the recurrent flares of Cyg X-1 (ref. 26) or the extended 'highs' (sometimes lasting for hundreds of days) exhibited by sources such as Cen X-3. In the context of a transient source association for Aql X-1, we note that the relatively soft flare X-ray spectrum⁶ suggests a generic relation to the class of 'long-duration' transients¹¹ (for example, A0620–00 A1524–62, and A1742–28), as opposed to the harder-spectral, shorter-lived transients exemplified by A1118–61 and A0535+26. The identification of the transient A0620–00 with a recurrent nova²⁶ and the resemblance of the long-term behaviour of Aql X-1 to both the transient X-ray sources and dwarf novae suggests that at least some members of the former group may arise from systems similar to the latter, but with the blue-dwarf binary component replaced by a neutron star or black hole.

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Variability of the BL Lacertae object AO0235+164

WE can summarise previous¹⁻³ discussion of the problem of excessive Compton scattering in variable compact non-thermal sources by saying that the small size implied by rapid variability leads to too large a probability that a given electron will Compton-scatter one of the emitted photons, leading to an excessive X-ray flux. The basic assumptions underlying these difficulties are that the time-variable radiation is produced by the incoherent synchrotron process by electrons with large pitch angles in randomly oriented magnetic fields, and that the objects are at the cosmological distances implied by their redshifts. We present here a crude model of the recently observed outburst⁴ of AO0235+164 in the near infrared and visible, where variability of up to 30% on time scales of 1 or 2 d has been noted. In our model the assumptions of large pitch angle and randomly oriented fields are relaxed⁵ and the model accords with our previous discussion³ of the low-frequency variability of the radio sources 3C454.3 and CTA 102.

The general picture is that of a strong dipolar magnetic field, with field lines of radius of curvature ρ , which is anchored to a spinning massive object, or 'spinar'. Electrons are accelerated by strong electric fields⁶ near the surface of the 'spinar', where $E \cdot B \neq 0$. They move freely along the magnetic field lines and acquire large momentum components parallel to B .

The observed 1–10-d variability of AO0235 in the near infrared and visible is then due to a burst of electrons of dimension $l \approx ct_v$, where t_v is the observed variability time scale, passing through a region of the magnetosphere in which the magnetic field is nearly tangent to the line of sight to the observer. The beaming angle θ of the burst is either the pitch angle ϕ of the electrons or the quantity l/ρ , whichever is greater. We show that for the parameters considered here $\phi < l/\rho$, so that $\theta \approx l/\rho$. Rieke *et al.*⁴ find that if the radiation at and near 10^{13} Hz were isotropic and at a distance given by the redshift 0.852, the luminosity in this spectral region would be 10^{47} erg s⁻¹. However, the small beaming angle θ implies that the total luminosity is actually $L_{\text{IR}} \approx 10^{47} \theta^2 / 4\pi \approx 10^{41} t_D^2 / \rho_V^2$, where ρ_V is the magnetic field line radius of curvature in light years, and $t_D = (86,400)^{-1} t_v$ is the variability time scale in days. The synchrotron power output of a single electron is $P_{\text{syn}} \approx 2 \times 10^{-15} B_{\perp}^2 \gamma^2$ erg s⁻¹, where $B_{\perp} = B \sin \phi \approx B \phi$, in gauss,

and γ is the Lorentz factor of the electrons. If N is the relativistic particle density, we have $L_{\text{IR}} \approx N l^3 P_{\text{syn}}$, and therefore

$$N \gamma^2 B_{\perp}^2 \approx 1.5 \times 10^9 t_D^{-1} \rho_V^{-2} \quad (1)$$

The synchrotron lifetime t_{syn} of the particles must be long enough for us to stand a reasonable chance of seeing them. With $t_{\text{syn}} \approx 20/(B_{\perp}^2 \gamma)$ in yr, $t_{\text{syn}} > \rho_V$ implies

$$B_{\perp}^2 \gamma < 20/\rho_V \quad (2)$$

Also, the electrons must radiate at predominantly 10^{13} Hz. Since we are interested in small pitch angles, we must remember that the usual expression for the 'critical frequency' ν_c for synchrotron radiation holds rigorously only for $\gamma \phi \gg 1$. But for our purposes, it suffices to set $\nu_c \approx 6 \times 10^6 B_{\perp} \gamma^2$ Hz for $\gamma \phi > 1$. The very small pitch angle case ($\gamma \phi < 1$) seems to be ruled out because of the high linear polarisations observed⁴, which are of the order of 10%. We set $\nu_c = 10^{13}$ Hz to obtain

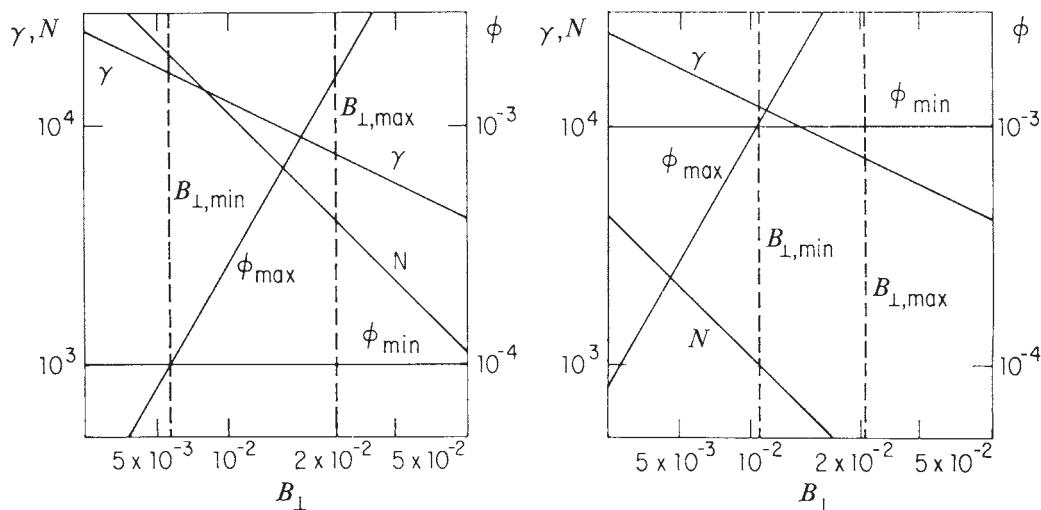
$$B_{\perp} \gamma^2 \approx 1.6 \times 10^6 \quad (3)$$

According to Epstein, small⁷ pitch angles give linear polarisations $P \lesssim \theta_R/\theta_B$, where θ_R is the angular width of the radiation about the magnetic field lines, so that $\theta_R \approx \phi + \gamma^{-1}$, and θ_B is a measure of the angular dispersion of the field direction in the source. But θ_B is just our 'beaming angle,' and so we have $P \lesssim (\phi + \gamma^{-1})/(l/\rho) \approx 300 \phi \rho_V / t_D$, since $\gamma \phi > 1$. The observed $P \approx 0.1$ then implies

$$\phi > 3 \times 10^{-4} t_D / \rho_V \quad (4)$$

We must now address the problem of excessive Compton-scattered X-ray flux, wherein we require the Compton-scattered X-ray luminosity L_{XR} to be less than L_{IR} . This is more restrictive than requiring the source to be undetected by Uhuru. We are aided by the fact^{3,5} that if ψ is the angle between the initial momenta of the electron and photon, the final scattered photon frequency is $\nu_{\text{XR}} \approx 2 \nu_{\text{IR}} \gamma^2 \sin^2(\frac{1}{2}\psi)$, and one can show³ that $L_{\text{IR}} > L_{\text{XR}}$ implies $1/8 N \sigma_T l \gamma^2 \psi^4 < 1$, for small ψ , where $\sigma_T \approx 6 \times 10^{-25}$ cm² is the Thompson cross section. ψ may be estimated by assuming that the field lines are concentric circles and then demonstrating geometrically that an average ψ is given by $\cos \psi = \rho \cos \phi / (\rho + 1/2l)$, or $\psi^2 \approx \phi^2 + l/\rho$. But

Fig. 1 The parameters of the model of AO0235+164 variable with a timescale of 1 d (left) and 10 d (right) for a magnetosphere of the size of 1 pc ($\rho_V = 3$).



since, as we have said, $\phi < l/\rho$, it follows that $\psi^2 \approx l/\rho$, and therefore the Compton-scattering condition becomes

$$N\gamma^2 < 5 \cdot 10^{14} \rho_V t_D^{-3} \quad (5)$$

There is one final constraint that we might impose. Since we wish the general shape of the magnetosphere to remain intact, we require that the magnetic field energy density be greater than the relativistic electron energy density in the burst, or $B^2/4\pi > N\gamma mc^2$. This gives

$$B^2 > 10^{-5} N\gamma \quad (6)$$

The relations (1)–(6) represent six constraints on the five unknowns B_1 , ϕ , N , γ and ρ_V (t_D is an observed quantity). From these conditions we obtain the following restrictions on the magnitudes of the parameters B_1 , ϕ , N and γ as functions of ρ_V and t_D :

$$1.8 \times 10^{-3} t_D \rho_V^{-2} < B_1 < 6 \times 10^{-2} \rho_V^{-2/3} \quad (7, 8)$$

$$3 \times 10^{-4} t_D \rho_V^{-1} < \phi < 1/3 t_D^{1/2} \rho_V B_1^{7/4} \quad (9, 10)$$

$$1.7 \times 10^4 t_D^{-1} \rho_V^{-4/3} < N < 1.7 \times 10^6 t_D^{-2} \quad (11, 12)$$

$$5 \times 10^3 \rho_V^{1/3} < \gamma < 3 \times 10^4 t_D^{-1/2} \rho_V \quad (13, 14)$$

The inequalities (7) and (8) are obtained from (5) and (2), respectively, by using (3) to express $\gamma \approx 1.3 \times 10^3 B_1^{-1/2}$ and then substituting into (1) to get $N \approx 10^3 t_D^{-1} \rho_V^{-2} B_1^{-1}$. The inequality (9) is just (4), and (10) follows by writing (6) as $\phi < B_1/B < B_1(10^{-5} N\gamma)^{-1/2}$ and then substituting for N and γ . The inequalities (7, 8) and (9, 10) are necessary and sufficient conditions for the validity of the model. We have left ρ_V as an 'external' parameter, since we wish to choose a value for it which is not much different from that in the authors' model⁶ for 3C454.3 and CTA 102, where $\rho_V = 1$.

Careful focus on a typical situation ($\rho_V = 3$) is depicted in Fig. 1, where the various parameters are presented now as functions of B_1 . The allowed ranges of B_1 are indicated by dashed lines. Solid lines show the corresponding values of N and γ , and the ranges of the pitch angles ϕ . The model 'pre-conditions' $\gamma\phi > 1$ and $\phi < l/\rho \approx 3 \times 10^{-3} t_D/\rho_V$ are satisfied here.

Thus we see that our crude model, with $\rho_V = 3$ typically, provides a description of the observed outbursts of AO0235 in the near infrared and visible, with total magnetic fields $B = B_1/\phi$ between 10 and 200 G, electron pitch angles between 10^{-1} and 10^{-3} , and Lorentz factors of 10^4 . The magnetosphere is thus about 1 pc in size, which gives total magnetic field energies $B^2 \rho^3$ of 3×10^{57} – 10^{60} erg, or between 10^3 and 3×10^6 solar rest masses. Total burst energies $N\gamma mc^2 f^3$ run between 3×10^{47} and 10^{51} erg.

Since the beaming angle is only 1–10 mrad, we would observe only about one event out of every 1,000. Correspondingly, outbursts of this sort are not often observed. A rough upper limit to the total output of AO0235 can be made by assuming that we observe one event every 10 yr, with a real rate of 100 yr^{-1} . If each outburst has an energy of 10^{49} erg, the average output is $3 \cdot 10^{43}$ erg s $^{-1}$, which is a typical quasar radio luminosity.

The question arises whether synchrotron radiation losses near the surface of the spinar, where the magnetic is much stronger, can substantially exceed the radiated energy in the outer magnetosphere, given by equation (1). A cursory calculation indicates that this is no problem, as long as the magnetic field does not exceed 10^5 G at the surface, taken to be 3×10^{16} cm from the centre⁵. If the field is not accurately dipolar near the surface, but is proportional to r^{-1} or r^{-2} there, there is no conflict with our choice of $\rho_V = 3$. However, if the field is accurately dipolar, then a model with $\rho_V = 0.3$ can be shown to be satisfactory.

With such a highly anisotropic electron distribution in pitch angle, there might be problems with instabilities caused by

generation of Alfvén waves in a thermal background plasma. This might very well have been the case in the early evolutionary stages of the magnetosphere. The coupling between the Alfvén waves and the beams of relativistic electrons would, on the other hand, mean that the thermal plasma would pick up momentum from the beams, and eventually be swept out of the magnetosphere. After a sufficiently long time, the magnetosphere would be practically evacuated and no further significant Alfvén wave production could occur. Our paper is concerned only with this latter phase.

Our model could also resolve the problem of the low-frequency variability of such radio sources as 3C454.3 and CTA 102, although coherent particle bunching is required. Cocke and Pacholczyk³ used $\rho_V = 1$ for these sources. Equally important, Sanders⁷ has shown that this general picture is capable of explaining in a natural way the apparent super-relativistic separation of the components of multiple radio sources, such as revealed by recent observations of 3C345 (ref. 9). Small pitch angles are necessary to Sander's discussion also, and expansion along the same direction in successive outbursts is provided by the stable orientation of magnetic axis.

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Uranus occults SAO158687

ON March 10, 1977, Uranus will occult SAO158687, a star of late spectral type with a visual magnitude of $\approx +9.5$ (refs 1,2). The occultation will be visible from land areas surrounding the Indian Ocean^{1,2}. From high-quality light curves of this event one can obtain temperature, pressure and number density profiles of the uranian atmosphere; information about its composition; and by combining occultation timings from several stations the precise diameter and oblateness of the planet can be found. For the atmospheres of Jupiter^{3–5} and Mars^{6–9} these quantities have been obtained from recent stellar occultations by these planets. This experience can be used to predict what can be learned from the forthcoming occultation by Uranus.

Although Uranus is several magnitudes brighter than SAO 158687 at visual wavelengths, the strong methane absorption bands in the far red and infrared spectrum of Uranus make the intensity of both objects nearly equal at these wavelengths². To see how to use the methane absorption bands to best advantage, we obtained the spectra of Uranus and SAO 158687 (Fig. 1). These spectra are meant to show the relative instrumental intensities of the two objects and have therefore not been calibrated on an absolute scale. The resolution of the spectra is 10 Å and the cutoff near 9,000 Å is due to a cutoff in

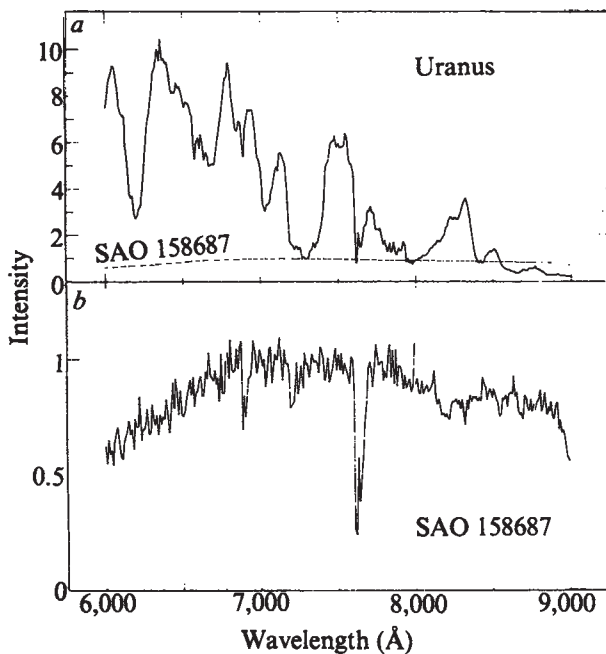


Fig. 1 Relative intensity of Uranus and SAO158687. Each spectrum was obtained with a 10-Å slit; the intensity scales are linear in the instrumental system. *a*, The two spectra on a common scale; *b*, an expanded version of the SAO158687 spectrum. In the deep methane band near 8,600 Å the stellar flux exceeds that of Uranus. The common absorption feature near 7,600 Å is the telluric Fraunhofer A band of molecular oxygen.

the instrumental response. The contribution from the sky background and dark current is negligible.

Table 1 lists four passbands where the relative intensities (integrated over the passband) of SAO158687 and Uranus are most nearly equal. Since the passbands are narrow, the ratio of these intensities is, for practical purposes, equal to the ratio of the photon fluxes f_*/f_u , where f_* and f_u are the photons $\text{cm}^{-2} \text{s}^{-1} \text{Å}^{-1}$ incident on the Earth's atmosphere from SAO158687 and Uranus, respectively. We have assumed SAO158687 to have UBVRI colours appropriate for a K5 main sequence star^{1,2,10} and computed the expected photon fluxes f_* (Table 1).

If observing conditions are good during the occultation, then the signal-noise ratio of the light curves should be limited by photon statistics. The latter give a fundamental limit for the signal-noise ratio achievable. We define $\phi(t)$ to be the flux from SAO158687 observed during the occultation, normalised to the unocculted intensity of the star. Hence $\phi(t)$ begins at 1.0 and decreases to 0.0 as the occultation proceeds. If ϵ_0 is the r.m.s. error in ϕ due to photon statistics for a 1-s integration, then ϵ_0 is equal to the square-root of the total photons detected from Uranus and the star, divided by the photons that would have been detected from the unocculted star

$$\epsilon_0 = \frac{(\bar{\phi}f_* + f_u)^{1/2}}{f_* \sqrt{(q\Delta\lambda A)}} \quad (1)$$

where $\bar{\phi}$ is the average value of $\phi(t)$ during a 1-s interval, A is the area (cm^2) of the telescope, $\Delta\lambda$ the passband of the filter (in

Å) and q the fraction of the photons incident on the Earth's atmosphere that are ultimately detected by the photomultiplier. The factor q includes the transmission of the Earth's atmosphere, telescope optics, photometer optics and the quantum efficiency of the photomultiplier. The quantity ϵ_0 has the dimensions of $\text{s}^{1/2}$, the actual r.m.s. error in ϕ for an arbitrary integration time of Δt is found by dividing ϵ_0 by $(\Delta t)^{1/2}$. In writing equation (1) we assume that the background counting rates from the photomultiplier itself and the sky are negligible. This may not be entirely true, since a quarter Moon will be only 17° away¹ during the occultation.

Values of ϵ_0 (Table 1) were computed for $\Delta t = 1 \text{ s}$, $q = 0.04$, $A = 6.6 \times 10^3 \text{ cm}^2$ (36-inch telescope) and $\bar{\phi} = 1/3$.

From Table 1 we see that low photon noise errors can be achieved by choosing appropriate passbands for observation. Such low errors could be realised in ideal conditions, but in practice changes in transparency, guiding errors, severe scintillation¹² and other problems could increase the effective noise in the signal above the level of photon noise.

From the photon noise levels in Table 1, we can calculate the r.m.s. error expected for the temperature profiles obtained from the occultation light curves, if we assume that the gravity gradient model³ applies to the uranian atmosphere. From the improved procedure of French *et al.*¹¹ for obtaining temperature profiles from occultation light curves, the minimum r.m.s. error in the scale height $\sigma(H)$, expressed as a fraction of the scale height H is

$$\frac{\sigma(H)}{H} \bigg|_{\min} \approx 1.5 \left(\frac{v_\perp}{H} \right)^{1/2} \epsilon_0 \quad (2)$$

where $v_\perp$ is the apparent velocity of SAO158687 perpendicular to the limb of Uranus. The scale height is related to the temperature T by $H = RT/\mu g$, where g is the gravitational acceleration, R the universal gas constant and μ the mean molecular weight of the atmosphere. At the occultation level we expect $T \sim 140 \text{ K}$ (ref. 13), hence $H \sim 65 \text{ km}$ (for $\mu \sim 2$). For this occultation $v_\perp \sim 12 \text{ km s}^{-1}$ (ref. 2). Even for the largest value of ϵ_0 in Table 1 (0.04), the fractional error in the scale height is only 2.6% (equation (2)). The minimum error in the temperature then will probably be dominated by uncertainties in the mean molecular weight.

At the occultation level the main constituents are likely to be H_2 , He and CH_4 . For solar composition the mixing ratio of CH_4 would be $\sim 7 \times 10^{-4}$ (ref. 13), which would leave H_2 and He as the major constituents. It may be possible to obtain a value for the helium number fraction $[\text{He}]/[\text{H}_2]$, from light curves at two wavelengths (6,200 and 8,600 Å, for example), as was done for Jupiter¹⁴, and for the $[\text{Ar}]/[\text{CO}_2]$ fraction in the case of Mars⁹. *A priori*, it is difficult to predict the expected accuracy of this measurement, since the accuracy will depend on the number and intensity of the 'spikes' in the light curves³.

The level of the atmosphere probed by the occultation corresponds to a number density n given approximately by the number density at 'half-light' for an ideal isothermal atmosphere¹⁵

$$n = \frac{\mathcal{L}}{v_{\text{STP}}} \left(\frac{T}{273 \text{ K}} \right) \left(\frac{H}{D} \right) \frac{1}{\sqrt{2\pi(R_p/H)}} \approx 6 \times 10^{13} \quad (3)$$

where $\mathcal{L}$ is Loschmidt's number, v_{STP} the refractivity of the

Table 1 Photon noise errors for selected passbands

Centre wavelength (Å)	Passband, $\Delta\lambda$ (Å)	Flux ratio, f_*/f_u	Flux† from SAO158687, f_*	r.m.s. photon noise error for a 1-s integration ϵ_0
6,200	100	0.14	0.2	0.04
7,300	200	0.5	0.4	0.011
8,000	300	0.7	0.5	0.007
8,600	400	1.2	0.6	0.006

†Photons $\text{cm}^{-2} \text{s}^{-1} \text{Å}^{-1}$ outside the Earth's atmosphere.

atmosphere at STP, D the distance to Uranus and R_p the radius of Uranus.

The 'central flash', observed during the occultation of ϵ Gem by Mars¹⁶, and used to determine the extinction of the martian atmosphere⁹, will probably not be observable, since the centre of the Uranus shadow is predicted to be off the edge of the Earth.

Observers of this occultation should record their data digitally. The shortest timescale of importance for the 'spikes' will be $\sim \delta D/2v_1 \approx 0.3$ s, where $\delta = 5.8 \times 10^{-4}$ arc s (ref. 2) is the angular diameter of SAO158687. Higher time resolution data recording would be desirable, but is not essential.

We plan to observe this occultation from Perth and over the Indian Ocean with the 91-cm airborne telescope¹⁴. Observers of this occultation interested in coordinating the analysis of their results should write to one of us. We acknowledge the support of grants from NASA.

Note added in proof: An updated prediction for this occultation can be found elsewhere¹⁷.

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Predicted and observed coronal structure

THE total eclipse of the Sun of October 23, 1976 was observed in excellent conditions from Victoria (Australia). The observation programme was designed to study the shape and structure of the corona: this structure is caused by magnetic fields. The corona's shape generally undergoes changes that follow the 11-yr cycle of solar activity. At the time of the eclipse the Sun's activity was near its minimum. In July the mean sunspot relative number was 2.1, in August 16.9, in September 13.4, in October 21.8, and in November 5.5. In these months the activity of the old cycle was still stronger than that of the new one, except in October. The high number of that month was produced mainly by the new cycle. It is not yet certain whether the minimum had been reached by the time of the eclipse. In any case, solar activity was low, but stronger than during most of the earlier minima. Therefore a typical 'minimum-corona' was to be expected, but not the shape of the 'undisturbed corona'. This shape is seldom observed, and was last seen on June 30, 1954, when the Sun was almost completely free of spots for three months before the eclipse¹.

The corona of October 23, 1976, consisted of two main parts completely different in character: a belt of large streamers, going up to heliographic latitudes of $b=60^\circ$, and the polar regions. As well as some weaker features, there were four large, broad and bright streamers, one in each of the four quadrants. Prominences were found at the

base of these streamers, at $b=+41^\circ$ and -35° on the E-limb and at $b=-43^\circ$ on the W-limb. The least developed of the four main streamers was that of the NW-quadrant, in which no prominence was observed. The axes of the streamers deviated strongly from the radial direction and were more or less parallel to the Sun's equator. On the poleside the streamers had a sharp edge. The heliographic latitude of that edge, measured at the Sun's limb, was $+64^\circ$ in the NE-quadrant, -55° in the SE-quadrant, -60° in the SW-quadrant and $+46^\circ$ in the NW-quadrant.

The polar regions were less bright than the main streamers. Each of the two polar cups contained a bundle of about 20 short and narrow streamers, the so-called polar plumes. Around the solar axis their direction was radial, becoming more and more inclined as the polar distance increases.

As the shape of the streamers is formed by the magnetic fields, K. H. Schatten attempted to predict the structure of the corona using the photospheric magnetic fields measured at the Hale Observatories (Mt Wilson) between September 13 and October 10. The result, a computer-drawn plot made on October 18 (ref. 2), is compared with the observations in Fig. 1, which is a sketch of the corona's structure based on photographs obtained at the eclipse. Generally the agreement between observed and predicted patterns is satisfactory. One huge equatorial streamer was

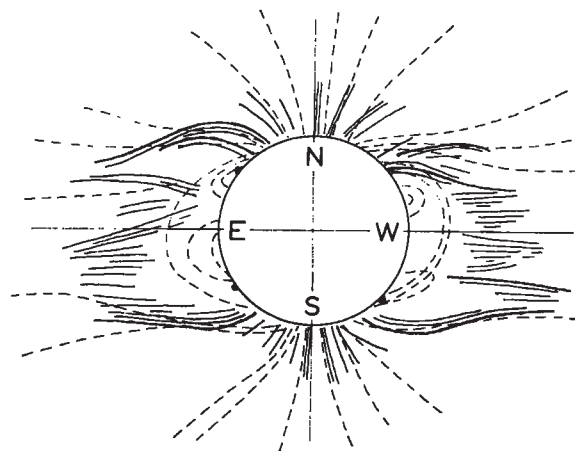


Fig. 1 The structure of the corona at the solar eclipse of October 23, 1976. Observations are represented by solid lines and predictions by dashed lines.

predicted for both the east and the west limbs of the Sun, as could be expected for an 'undisturbed' corona. In fact, there were two streamers on each limb, one in each hemisphere, representing the 'minimum-type' rather than the 'undisturbed' corona. The dipolar field represents the polar plumes well. The positions at which the edges of the long streamers limit the polar regions were correctly predicted for the NE- and SW-quadrants. In the SE- and SW-quadrants, however, the observed heliographic latitude of the edges was about 20° smaller than the prediction.

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Improved and extended theory of the Berthelot tube method of studying liquids under tensile stress

ALTHOUGH the Berthelot tube method of subjecting a liquid to tension has been used intermittently for several years, there is no published theory which enables us to calculate the value of the tension at any stage before the breaking, that is, rupture of the enclosed liquid. We present here such a theory, giving good agreement with the experimental values of the tension obtained directly using a transducer method. In the past, two theories of the method have been given but these only enable us to calculate values of the breaking tension; first, the theory of Vincent and Simmonds (method 1), which we shall show to contain a serious error and second, that of Temperley and Chambers (method 2). These two methods have been discussed by Chapman *et al.*¹

A liquid can withstand tension due to its ability to exist in a metastable phase. This has applications in work on cavitation, in the design of such things as ship propellers and in the field of lubrication. In its metastable phase a liquid is superheated and this is the basis of the bubble chamber where the path of an ionising particle is shown as a trail of vapour bubbles as it passes through a superheated liquid; the ions produced by the high energy particle act as nuclei on which these bubbles can form. Columns of sap rise to the top of very tall trees due to the existence of tension in the sap column; tensions of > 50 atm have been found in mangrove trees.

The Berthelot tube method is as follows. A sealed tube, usually of glass or steel, is almost completely filled with the test liquid while the remaining volume is occupied by air and liquid vapour. On heating, the liquid expands to fill the whole tube and positive pressures are generated in the liquid. If, next, the tube is allowed to cool slowly, the liquid continues to fill the tube and adhere to its inside walls; during cooling the positive pressure inside decreases to zero and then changes to a negative pressure (tension) until the liquid eventually breaks with the onset of cavitation. At this 'break' there is a sudden increase in the volume of the tube as the internal tension is released. Richards and Trevena² have followed these pressure changes directly using a pressure transducer and have obtained some experimental (pressure, temperature, that is) (p, T) curves for water (Fig. 1).

Our proposed theory is as follows. If we consider one of these (p, T) curves (say curve *a*) then the point on the curve where the pressure is zero corresponds to the state in which both the tube and enclosed liquid are in an unstressed condition. Let

V_0 and T_0 be respectively the internal volume of the tube and the temperature at this stage (stage 1). The tube and its contents are then cooled so as to follow the curve to a lower temperature in the tension region where the internal volume of the tube is V_1 and the temperature T_1 ($< T_0$) (stage 2). At this stage tension F is generated in the enclosed liquid and both the liquid and tube are in a state of stress. At this stage we then consider what the unstressed volumes of the liquid and tube would be at the existing temperature T_1 . The unstressed volume W_1 of the liquid would be $< V_1$ and the unstressed volume of the tube would be $V_1 - \Delta V_1$ (stage 3). (This is precisely what would happen if the enclosed liquid were to actually break at T_1 .) We let ρ_0 and ρ_1 denote the densities of the unstressed liquid at the temperatures T_0 and T_1 (stages 1 and 3, respectively).

For the unstressed liquid in stages 1 and 3

$$V_0 \rho_0 = W_1 \rho_1 \quad (1)$$

Next consider stages 2 and 3 for the liquid at T_1 . The volume of the liquid is V_1 under a tension F and W_1 under zero tension ($W_1 < V_1$). Thus, if K_1 is the bulk modulus of the liquid

$$K_1 = -\frac{F}{(V_1 - W_1)/W_1} \quad (2)$$

Similarly, let us consider stages 2 and 3 for the tube at T_1 . Its inside volume is V_1 , under an internal tension F , increasing to $V_1 + \Delta V_1$ under zero internal tension, when the liquid breaks. Thus, if K_s is the bulk modulus of the material of the tube,

$$K_s = -\frac{F}{\Delta V_1/(V_1 + \Delta V_1)} \quad (3)$$

Finally, we consider stages 1 and 3 for the tube; since the tube is unstressed in both cases,

$$V_1 + \Delta V_1 = V_0(1 - 3\alpha\Delta T_1) \quad (4)$$

where $\Delta T_1 = T_0 - T_1$ and α is the linear coefficient of expansion of the material of the tube.

Equations (1)–(4) give the 'basic physics' of the method and can be solved to give an expression for the tension F

$$F = \frac{\rho_0/\rho_1 - (1 - 3\alpha\Delta T_1)}{\rho_0/\rho_1 K_1 + (1 - 3\alpha\Delta T_1)/K_s} \quad (5)$$

in terms of the known or measurable quantities α , ΔT_1 , ρ_0 , ρ_1 , K_1 and K_s .

Suppose that we return to our initial stage 1 and move up the curve *a* of Fig. 1 to a point in the pressure region; let the pressure inside the tube at this stage be p and the corresponding temperature T_2 ($> T_0$). We can then obtain a value for the pressure p generated inside the tube at the temperature T_2

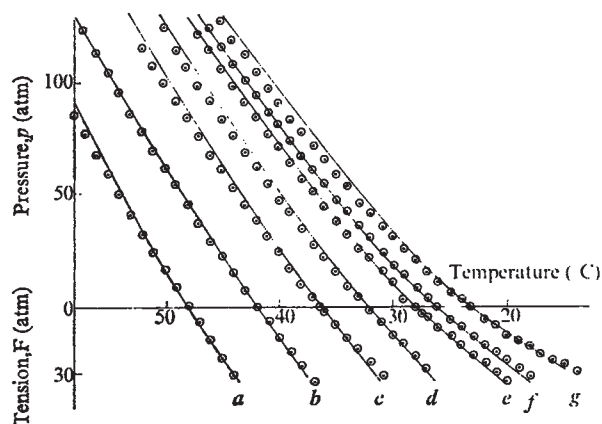
$$p = \frac{(1 + 3\alpha\Delta T_2) - \rho_0/\rho_2}{\rho_0/\rho_2 K_1 + (1 + 3\alpha\Delta T_2)/K_s} \quad (6)$$

where $\Delta T_2 = T_2 - T_0$ and ρ_2 is the density of the unstressed liquid at the temperature T_2 .

The present theory enables us to calculate the tension or pressure in the enclosed liquid at any stage of the process, using equations (5) and (6), thus improving on previous theories of the Berthelot method which only give an equation for the value of the actual breaking tension.

From integral values of ΔT_1 and ΔT_2 we can compute the values of F and p from equations (5) and (6) using the appropriate values of the other quantities involved as obtained from

Fig. 1 Full lines: experimental (p, T) curves obtained by Richards and Trevena for water in a steel Berthelot tube. $\odot$, F and p values calculated using the present theory.



tables. These calculated values are shown in Fig. 1 and the agreement between theory and experiment is satisfactory.

In method 1, Vincent and Simmonds write

$$V_b = V_r [1 + 3\alpha(T_r - T_b)] \quad (7)$$

where V_r is the volume at the filling temperature T_r and V_b that immediately after the break at the temperature T_b when the tube is in an unstressed state. But an equation such as (7) can only be applied provided the tube is in the same state of stress at both T_r and T_b . Chapman *et al.*¹ have, however, shown that pressures of 50–100 atm exist in a Berthelot tube at T_r . Thus the theory of method 1 is incorrect since it only takes into account volume changes due to temperature differences and ignores those due to the change in the state of stress of the tube; our theory takes both of these factors into account. Method 2 predicts values for the breaking tension which agree quite well with the experimental values obtained directly from the transducer measurements of Richards and Trevena. One great advantage, however, of our theory is that equation (5) enables us to calculate the tension inside the tube at any stage before rupture; methods 1 and 2 only enable us to calculate the values of the actual breaking tension (and method 1 does that incorrectly). Also, our theory is in satisfactory agreement with experiment, in both pressure and tension regions (Fig. 1).

We hope that we have corrected some previous errors, and both extended the Berthelot tube theory and put it on a firmer foundation.

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Ion-pair clusters and excited-state yields in radiolysis

WHEN β or γ rays are absorbed in matter, sparse tracks of ion pairs are produced: the secondary electrons sometimes have enough energy to produce further ion pairs in small clusters. Distributions of cluster size in a cloud chamber were measured by Wilson as long ago as 1923 (ref. 1); reinterpretation² of his and other results shows that nearly two thirds of the ion pairs occur in clusters. Similar behaviour should occur in other phases, but little is yet known of the role of clusters in radiolysis. In hydrocarbons, the geminate recombination of radical ions is a major process: most theoretical descriptions have dealt only with single ion pairs³. Since most of the ion pairs recombine very quickly, the clusters will rapidly become single pairs, so the approximation is probably quite good for scavenging studies. If we consider recombination products, especially the yields of singlet and triplet excited states, cluster formation must be taken into account⁴.

Measurements made by pulse radiolysis^{5–7} of yields of excited singlet and triplet states, $^1M^*$ and $^3M^*$, in alkanes and benzene derivatives containing added aromatic hydrocarbons (M) have shown triplet-to-singlet ratios, T/S, ranging between 1 and 2. Hereafter 'triplet' and 'singlet' will usually refer to the ion pairs. Though the ions are so far apart (~ 50 – 100 Å) for most of the life of a pair that the unpaired electrons do not interact, one can still speak of singlet or triplet character^{8,9}.

A single ion pair will normally be formed in a singlet state: if recombination is fast enough, only $^1M^*$ will be produced⁸.

Single triplet pairs will be formed if the secondary electron has sufficient energy to excite another molecule to a triplet state by electron exchange¹ (process A). In clusters, some triplets will be formed by cross-recombination (process B). Calculation of T/S for B is a surprisingly difficult mathematical problem; however, combining the results of Atkins and Lambert¹⁰ with the spur size distribution² gives 0.34.

Initial spin correlation in an ion pair will decay over some tens of nanoseconds⁹; the rate of decay and its final extent can be changed by magnetic fields^{8,9}. In the simple case of a single pair, initially singlet, the final state at zero field consists of one quarter singlets, three quarters triplets: in a strong magnetic field, the singlet can only interact with T_0 : the $T \pm$ components become inaccessible because of their Zeeman energy: the final state is half singlet, so the field should increase the yield of $^1M^*$ and of fluorescence by a factor of 2 at long times.

By the single-photon counting method, I measured scintillation pulse shapes for solutions of *para*-terphenyl, anthracene (and their perdeutero-analogues), biphenyl and perylene in a number of alkanes and benzene: the enhancement ratios at long times (~ 100 ns after ionisation) are in the range 1.30–1.45 for alkanes: there is little or no dependence on the nature of the solute. These are tentative figures: the limiting values may not be reached, or the yields of $^1M^*$ and $^3M^*$ may be influenced by energy level densities (Frank-Condon factors)⁹. However, in both cases one would expect to see differences between solutes. Therefore, I propose that these low values are due to scavenging of initially triplet ion pairs. Triplets decay into singlets reducing the net effect; if the initial T/S were 3, there would be no net change and the field effect would disappear: the above range corresponds to initial T/S ratios between 1.1 and 0.7. This interpretation is supported by the case of benzene solutions, where the enhancement is only 1.09 (T/S ~ 2.15). The benzene triplet state lies well below those of the alkanes, so that process A will be more important.

Total yields of $^1M^*$ and $^3M^*$ (refs 5–7) are less easy to interpret: the excitation mechanisms are still being debated; in particular it has been proposed⁶ that singlet excitation transfer from the solvent is the main source of $^1M^*$ rather than ion recombination⁷. Also, ion recombination is slow enough that significant decay of the spin correlation occurs before recombination. The extent of this can be estimated from measurements of enhancement of fluorescence and is about 10% for solutions in cyclohexane¹², that is a measured T/S ratio of yields of 1, would correspond to an initial ratio of 0.82. Precise calculations are not possible at present, but there appears to be no inconsistency between the magnetic effect data and the measurements with pulse radiolysis, provided that recombination is the major source of excited states in alkane solutions.

In striking contrast is the work of Lipsky *et al.*^{13,14} who have measured the fluorescence yields of the alkanes themselves, avoiding complications produced by solutes. For bicyclohexyl, they estimate that T/S ≤ 0.25 ; this is an initial value since recombination in the alkanes is extremely fast (picoseconds).

The discrepancy can be explained by noting that processes A and B, in which an overall singlet spur produces two or more triplets, are both reversible; for example, in a two-pair cluster a triplet ion pair may recombine to a triplet molecule, which can exchange spins with the second electron as it returns to the neighbouring cation (being quenched in the process). Scavenging of electrons by a solute will allow more time for cation and triplet or two cations to separate, even though ion recombination may still occur in $\lesssim 1$ ns. For solutions, the enhancement by the field varies considerably among the alkanes; this can hardly be due to process A; there seems to be a correlation with positive charge mobility—probably an effect of scavenging rate. On this basis, one would expect larger overall yields of excited states in benzene etc., and this is found^{13,11}; process A, however, also contributes to this.

These results demonstrate the usefulness of the magnetic field effect for studying geminate ion recombination: measurements at long times are free from interference from energy

transfer, which must be complete in a few nanoseconds; free ions only contribute to the background which is subtracted out.

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Resistivity and the continental crust in southern Africa

DURING the last decade many deep electrical soundings have been made over the major Precambrian tectonic provinces of southern Africa^{1,2}. The results (Van Zijl, submitted for publication) show that there are marked differences in the electrical properties between Archaean cratonic terrains (Kaapvaal and Rhodesian cratons) and mobile belts (Limpopo, Namaqua and Damara). The ages of the cratons are >2,600 Myr, whereas the dominant ages of the mobile belts are 2,700, 1,000 and 500 Myr, respectively³. In the discussion of crust here, the overburden or cover rocks of the various tectonic provinces have not been considered.

Cratonic terrains are characterised by a highly resistive zone extending from the surface down to a depth of 5–10 km depending on the locality. The average resistivity of this zone is about 100,000 Ω m with values varying between 30,000 and 500,000 Ω m. In some areas the highly resistive layer is overlain by a more conductive layer with an average resistivity of about 55,000 Ω m and a variable thickness.

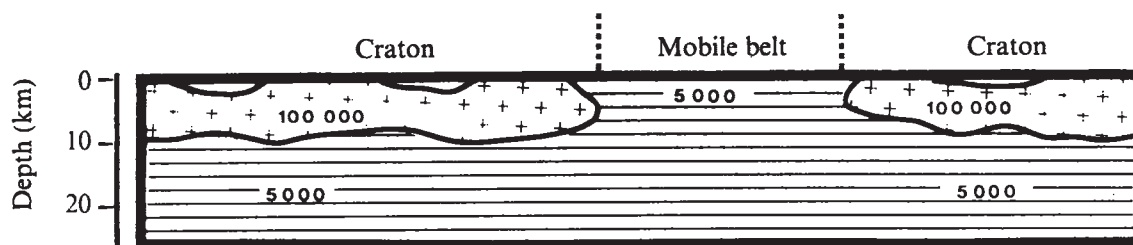
In contrast, the prominent highly resistive zone associated with the cratons is completely absent in the mobile belts. Instead, a thick moderately resistive zone with a resistivity ranging from 2,000 to 8,000 Ω m (average 5,000 Ω m) extending to a depth of 25–30 km is recognised. An important feature of this zone, deduced from closely spaced soundings, is that it continues laterally into the craton where it underlies the highly resistive layer. The electrical model is shown schematically in Fig. 1.

Correlation of the relevant electrical horizons with the results of geological investigations^{4–10} shows the following relationships. First, the uppermost 5,000 Ω m layer observed in cratonic areas is due to the presence of metamorphic rocks of supracrustal origin. Second, the highly resistive zone in cratonic terrains is associated with vast amounts of granitoid material in the form of granitic intrusions, gneisses and migmatites. Third, the 5,000- Ω m layer of the mobile belts can be attributed to the presence of intensely deformed and highly metamorphosed rocks exhibiting features of upper amphibolite and granulite facies metamorphism. Fourth, the lower crust is metamorphic as deduced from the continuation of the 5,000- Ω m layer under the highly resistive layer associated with the cratons.

The view that the stable continental crust of cratonic areas is made up of a lower crust of high-grade metamorphic rocks underlying a more granitic upper crust which also contains remnants of supracrustal metamorphics, is not new. It was proposed, on geological grounds, by Wegmann¹¹ as early as 1935 and is supported by numerous later studies^{12–18}. Geophysical arguments based particularly on heat flow¹³, seismic¹⁹ and gravity²⁰ investigations agree with this model. Basically, the lower crust is considered to consist of rocks which have largely been depleted of lighter granitic materials, volatiles and radioactive elements, leaving behind a refractory residuum. The metamorphic layer grades upwards into a zone in the upper crust consisting predominantly of granitic material occurring in the form of intrusions, gneisses and migmatites which were mainly mobilised from melts originating from the lower crust. It has often been noted that rocks of this zone both intrude and form the basement to supracrustal metamorphic rocks^{4,14,31}. The extremely high resistivities associated with the granitoids indicate that the rocks are solid, strongly consolidated and largely unfractured.

The suggested correlation of the highly resistive layer with cratonic upper crust is significant as it provides a means of determining the extent of cratonic material even into adjoining mobile belts provided the grade of metamorphism remains low enough to prevent the migration of the granitoid material to higher tectonic levels¹¹. Results to date indicate that the distribution of the ancient cratonic layer was indeed extensive and give the impression that this layer was broken up by the mobile belts studied. The Limpopo and Namaqua mobile belts, where a highly resistive cratonic material is bound on both sides of a core of moderately resistive highly metamorphosed rocks, are good examples. The view that the southern African mobile belts represent reworked and reconstituted cratonic crust^{8,9,10,11,18} is thus supported by the electrical studies. Further evidence comes from complementary electrical information derived from the results of magnetometer array studies^{21,22} which show that these mobile belts are not underlain by a highly conductive lithosphere as would be expected if they represented the traces of subduction zones involving oceanic crust. The mobile belts are thus regarded as being of ensialic origin, as suggested also by geological evidence^{8,10}.

Fig. 1 Schematic resistivity model of the Precambrian continental crust obtained from direct current Schlumberger electrical soundings. The average resistivities (Ω m) of the electrical horizons are shown. The highly resistive zones are associated with granitoid material (—) in cratonic terrains, whereas the more conductive zone is correlated with intensely deformed high-grade metamorphic rocks (---). The isolated synforms (white) overlying the granitoid material represent supracrustal metamorphics. Cover rocks are not shown.



The resistivity-derived model indicates that the floor of the mobile belts is formed by high-grade metamorphic rocks which are probably of deep crustal origin. In accordance with the views of Kröner⁹ and Shackleton¹⁸ it seems that the crust has become decratonised with time and is weakened, not stiffened, by events of deformation and metamorphism which have often been polycyclic.

The electrical model can be extended when account is taken of the absolute values of resistivity obtained from deep sounding results. From available temperature gradient estimates²³, it can safely be assumed that the temperature at a depth of 25–30 km is too low for conduction through minerals to be important²⁴ and that crustal rocks conduct electricity by virtue of interconnected cracks filled with water²⁴. The space occupied by water can be calculated if the rock resistivity and the water resistivity are known²⁵. While the latter parameter cannot be measured directly at great depths, it can be estimated by the extrapolation of water resistivities measured near the surface. From measurements on samples from boreholes and in mines (van Zijl, submitted for publication) a value of 1 Ω m has been assumed for water at depth. On this basis the porosity of the upper crustal cratonic layer is <0.5%, which substantiates its basically solid and unfractured nature as noted earlier. The mobile belts, on the other hand, have considerably larger porosities, varying from 3% near the surface to about 1% at a depth of 25 km, implying that these rocks are cracked and fractured and consequently weakened, not only in mobile belt areas but also in the lower crust under the cratons. Various studies have indicated that pore pressure is high during metamorphism and that a high degree of crack space is required^{24,26}. These observations considered jointly with the intensely deformed nature of these rocks, may explain the origin of the cracks. The depth to which the connectivity of crack space continues will depend on the temperature at which permanent deformation of the rock minerals occurs. Experimental work²⁷ has shown that temperatures in excess of 500 °C are needed, which correspond to depths greater than 30 km in normal crustal situations. When viewed over geological periods, the interconnectivity of cracks, which come right up to the surface in mobile belts, implies hydrostatic pore pressure²⁸ and a decrease of porosity with depth²⁵. This supposition is supported by the resistivity results over mobile belts, as noted earlier. The origin for the water in the crust may thus be meteoric²⁹. The several-fold increase of the porosity at the boundary of cratonic layer and the underlying metamorphic zone may account for the low velocity layer found at this depth in the Canadian Shield³⁰, a region geologically similar to the areas studied in southern Africa.

The suggestion that free water is available at all levels in the crust has many implications regarding physical processes and geochemical effects. The variation of heat flux and the nature of the crust–mantle boundary are only two examples.

Thermal gradients²³ and heat flow estimates (A. E. Carte, personal communication) from mobile belts appear to be anomalously high when compared to cratonic terrains, especially when the lower heat production of highly metamorphosed rocks¹³ is taken into account. This paradox may possibly be explained by the transport of heat by the preferential upward movement of water through the cracked metamorphic zone and its concentration in mobile belts. Regarding the crust–mantle transition zone, electrical sounding results sometimes show the presence of a conductive layer at this depth which is interpreted at present as being due to hydration processes of mantle rock (van Zijl *et al.*, in preparation).

It is clear that electrical resistivity is intimately associated with both the nature and the structure of the continental crust; its usefulness as a tool in crustal investigation critically

depends on the quality of the data obtained from electrical investigations.

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Age and position of the Ellsworth Mountains crustal fragment, Antarctica

THE present geographical position of the Ellsworth Mountains is geologically anomalous on account of their stratigraphy, which does not relate to western Antarctica, their structural trend, approximately perpendicular to the trend in adjacent regions, and their relief, the greatest, and possibly the youngest, in Antarctica. These factors suggest that the Ellsworth Mountains are not in their original position but have moved, probably during the Cainozoic.

During a radio-echo ice-depth-sounding flight on January 22, 1975 from the remote American 'Siple' station in inland Antarctica, a British Antarctic Survey aircraft landed beside two small nunataks at the edge of the Ronne Ice Shelf at 77°02'S, 78°20'W (Fig. 1). In 1947, Ronne¹ sighted a nunatak at 77°40'S, 79°00'W which he named Mount Haag. As we found no other nunataks within 180 km, these are considered to be Mount Haag, now renamed Haag Nunataks.

Under the direction of Dr C. W. M. Swithinbank, seven specimens of the *in situ* rock types were collected comprising four granodiorite–gneisses, one hornblende schist, one quartzo-feldspathic gneiss and one acid vein cutting gneiss. All but one of the rocks show acid veining to some extent.

The granodiorite–gneisses generally have a strong foliation marked by biotite (<10%) which is frequently altered to chlorite and associated with hornblende (<3%). Quartz crystals are mostly strained, often fractured and may occur as single crystals or in a mosaic. Oligo-andesine, usually heavily sericitised, is predominant, and potash feldspar is present mainly as micropertite of a string or bead type, but microcline with characteristic twinning also occurs.

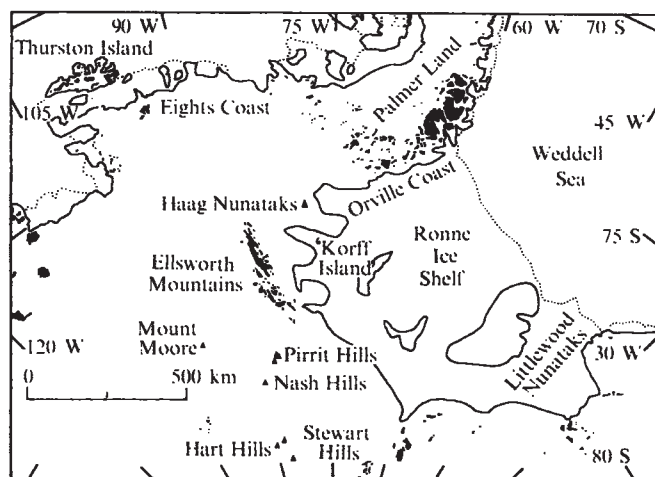


Fig. 1 Map of the Ronne Ice Shelf and its environs; areas of exposed rock are shown in black.

Potash feldspars rarely show any alteration, and clear untwinned feldspars are probably cryptoperthite. Accessory minerals include pistacite, sphene, allanite and ore minerals.

The hornblende schist has a granoblastic texture and comprises about 29% green hornblende, 39% andesine with quartz, biotite, accessory sphene and some ore. Acid veinlets and augen in the schist are much richer in quartz and contain some potash feldspar whereas hornblende is a minor constituent. The quartzo-feldspathic gneiss consists of microcline augen, quartz, perthite, plagioclase and biotite. The specimen of an acid vein in gneiss is essentially similar in the vein part to the quartzo-feldspathic gneiss and to the grandodiorite-gneisses in the gneissic part.

These rocks seem to have been regionally metamorphosed to amphibolite facies and suffered synorogenic invasion by acid veins. No observations of field relationships could be made but colour photographs of the outcrop suggest that some of the rocks may be migmatites.

This suite of rocks is typical of many regional metamorphic terrains of Antarctica but the nearest known outcrops of similar rocks are along the Eights Coast and in the eastern part of Thurston Island² or in the coastal regions of northern Palmer Land³. 'Granites intrusive into gneisses' were reported, however, at 81°53'S, 69°20'W by the 'Ellsworth' station-Byrd station traverse party⁴. The nearest outcrops inland of the Orville Coast⁵ and in the Ellsworth Mountains⁶ comprise sedimentary rocks, those in the Nash and Pirrit Hills contain granitic intrusions, and Mount Moore and the Stewart Hills consist of phyllite and

schist⁷. Behrendt⁸ reported a nunatak to the west of 'Korff Island' but this is now considered to be an ice fall (C. W. M. Swithinbank, personal communication).

K-Ar determinations on two gneisses from Haag Nunataks have yielded the results shown in Table 1.

The concordant dates obtained from separates of hornblende and co-existing biotite provide a minimum age for the last major regional metamorphism affecting the gneisses. The lower apparent ages obtained from the potash feldspars and plagioclase are due to argon loss from these less retentive minerals, and are not necessarily geologically significant. It is interesting that a date of 1,044 Myr (ref. 9) has been obtained from rhyolites in the Littlewood Nunataks and may reflect some association in time and space with the gneisses from Haag Nunataks.

Schopf¹⁰ suggested that the Ellsworth Mountains lie on a fragment of continental crust which has moved to its present position from the north-east. Assuming Haag Nunataks are an up-faulted slice of the metamorphic basement underlying the Ellsworth Mountains, these dates provide additional evidence to suggest that the Ellsworth Mountains area was originally along the western margin of the Antarctic plate. Apart from a deep subglacial trough between the Ellsworth Mountains and the Orville Coast¹¹ which may continue eastward to the north of Haag Nunataks, there are insufficient data to delimit the margins of this fragment of continental crust until the bedrock topography of the Weddell Sea and its environs is known. The structure and stratigraphy of the Ellsworth Mountains¹¹ and the ages from Haag Nunataks suggest an original position for this fragment of continental crust westward of the Littlewood Nunataks (where there is a bathymetric depression), but an accurate palaeogeographical reconstruction of this region must await palaeomagnetic data from the Weddell Sea floor.

We thank Dr C. W. M. Swithinbank for the opportunity to study these specimens. The flight to Haag Nunatak was made possible through the provision of aviation fuel and accommodation by the NSF at 'Siple' station.

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Table 1 Analytical data

Sample no.	Material analysed	% K	Vol. rad. ⁴⁰ Ar (nl g ⁻¹)	Age (Myr)
E.4690.1	Hornblende	1.04	55.79	1,018 ± 28
	Biotite	6.39	332.3	991 ± 22
	K-feldspar	10.4	381.5	745 ± 18
	Plagioclase	0.553	16.45	628 ± 18
E.4690.2	Hornblende	1.01	55.30	1,031 ± 14
	Biotite	6.79	358.3	1,002 ± 24
	K-feldspar	9.99	356.2	731 ± 18
	Plagioclase	0.676	18.86	595 ± 16

Ages have been calculated with $\lambda_p = 4.72 \times 10^{-10} \text{ yr}^{-1}$, $\lambda_e = 0.584 \times 10^{-10} \text{ yr}^{-1}$ and $^{40}\text{K}/\text{K} = 0.0119 \text{ at. \%}$.

Errors are quoted at the 2 σ level and take into account uncertainties in the mass spectrometer ratio measurements, spike calibration, atmospheric argon correction and potassium determinations.

Argon concentration was determined by standard isotope dilution techniques and potassium was determined in quadruplicate by flame photometry.

Geochronological evidence of Hercynian activity in Newfoundland

A RECONSTRUCTION of the continental blocks for the central and North Atlantic has led to the widespread acceptance of the continuity of the Palaeozoic orogenic belts on both sides of the Atlantic. A reconstruction of the land masses during the Carboniferous and Permian shows the Variscan Belt of Europe, the Mauritanides of Morocco, and the

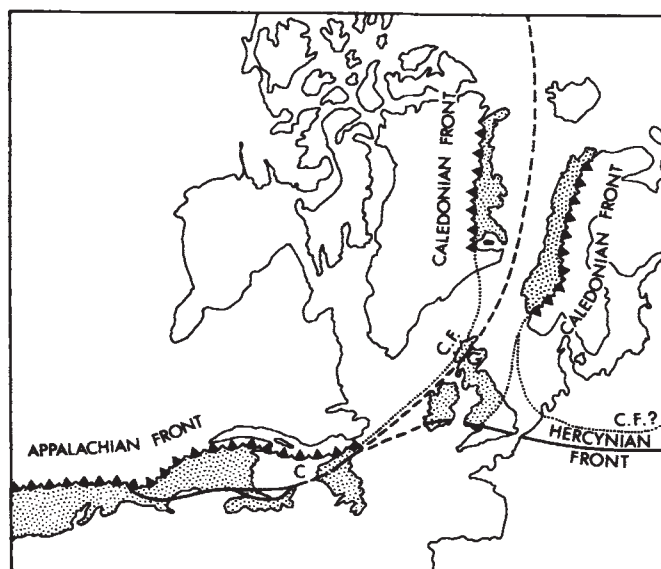


Fig. 1 Reconstruction of continental blocks around the North Atlantic during the Permian (after Holmes³). C. F., Caledonian Front of northern Scotland; G, Great Glen Fault; C, Cabot Fault.

Southern Appalachians as being continuous'. Although the Hercynian Front has now been recognised in New Brunswick² the situation in Newfoundland is unclear. We suggest, on the basis of the geochemical data outlined in Table 1, that the continuation of these belts can be interpolated between New Brunswick and south-west England to include at least the Central Mobile Belt and the Avalon Zone of Newfoundland. We find the earlier correlation by Holmes³ particularly attractive in view of our new isotopic data, a correlation which extends the Hercynian Front westwards from south-west England and Wales into Canada, where it passes along the Cabot Fault into the New Brunswick seaboard and the Bay of Fundy (Fig. 1). Cherkis *et al.*⁴ link the Charlie Gibbs Fracture Zone to the Hercynian Front on both the European and North American continents, and have recently offered new evidence for the emergence of the front in Newfoundland⁵. The intersection of the Charlie Gibbs Fracture Zone and

the continental shelf of Newfoundland lies about 100 km east of the Cabot Fault, so that their delineation of the front lies further to the south than the one outlined here.

The four granites listed in Table 1 and shown in Fig. 2 record either intrusive or deformational events of Carboniferous age. Three of these granites are emplaced into the crystalline margins of the Central Mobile Belt, while the St Lawrence granite is intrusive into Palaeozoic and Late Precambrian sedimentary and volcanic sequences of the Avalon Zone. The St Lawrence granite, a non-foliated, high-level, peralkaline body associated with fluorite mineralisation, intrudes a zone of weakness marked by a series of north-south trending folds, north-east trending synclines, and high-angle thrusts that run parallel to the fold axes⁶. The remainder of the granites are either sheared or cataclastically affected by regional deformation. The Lockers Bay granite, a megacrystic microcline variety, is intimately associated with the Dover Fault, a major tectonic discontinuity that separates the northern part of the Central Mobile Belt from the Avalon Zone. This granite was considered to be Hadrynian in age, or older⁷. To the north, in the Burlington Peninsula area, the regional deformation which post-dates the intrusion of the LaScie intrusive complex and the Cape Brulé porphyry has been attributed to either pre-Ordovician^{8,9} or post-Lower Ordovician-Devonian movements¹⁰. At least two phases of deformation have affected the LaScie complex; using some of the geochronological evidence presented here, DeGrace *et al.*¹¹ concluded they "are probably both Hercynian deformations".

Widespread basement reactivation in Europe during late Carboniferous times (Westphalian-Stephanian) produced many of the granitic masses of the Bohemian Massif, the Iberian Peninsula, Morocco, the Schwarzwald and the Massif Central. As a consequence of partial melting of a thickened continental crust (brought about by continent-continent collision of the northern and southern European plates) these granites were enriched in potassium relative to their more sodic counterparts formed during the Caledonian Orogeny¹². Similarly, the granites listed in Table 1 seem to be quite different to granites of Devonian age that we have dated from Newfoundland. The marked contrast of the initial ratios shown in Table 1 to the lower ratios (0.704-0.709) for many of the Acadian granites is entirely consistent with derivation of the younger granites by partial melting of continental crust.

Variations in age, deformation and metamorphism along the length of the Caledonian-Appalachian-Hercynian

Table 1 Whole-rock Rb-Sr isochron data*

Locality	Petrographic and geochemical notes	Apparent age (Myr)	Initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio	No. of points
LaScie Intrusive Complex	Fine- to medium-grained alkali granite; mainly quartz, orthoclase; rare plagioclase, amphibole and opaques; traces of epidote and carbonate—evidence for cataclasis	315 ± 25	0.715 ± 0.012	5
Cape Brulé porphyry	Feldspar and embayed quartz phenocrysts set in fine-grained groundmass; alteration products, sericite, chlorite; traces of calcite, fluorite; some evidence for cataclasis	325 ± 14	0.7145 ± 0.0018	5
Lockers Bay granite	Megacrystic microcline, biotite granite; strongly schistose—microcline phenocrysts some flattened; granulation of quartz and feldspars; epidote common	300 ± 18	0.7146 ± 0.0013	6
St Lawrence granite	Red to pink, medium- to fine-grained; mainly quartz, orthoclase, albite, with minor riebeckite, aegerine, biotite, fluorite, magnetite and haematite; perthitic and granophyric textures common	315 ± 10	0.722 ± 0.005	4

* $\lambda^{87}\text{Rb} = 1.47 \times 10^{-11} \text{ yr}^{-1}$.
All errors given at the 2 σ level.

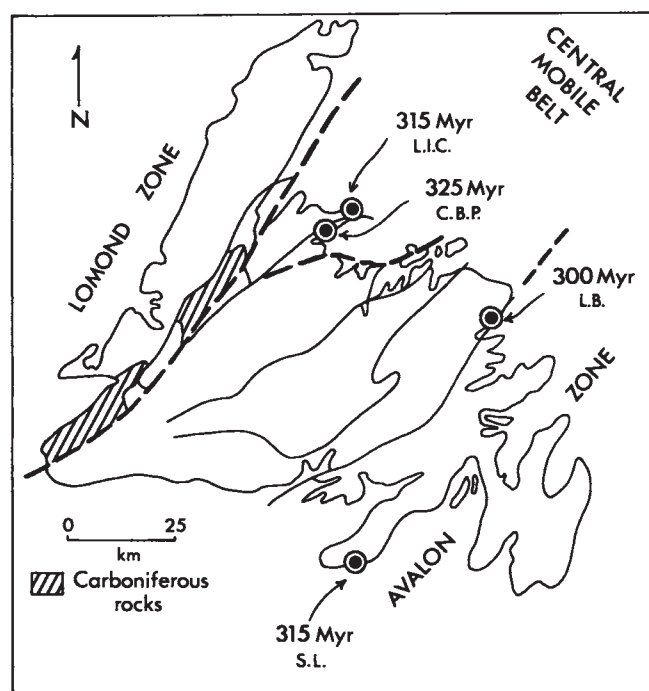


Fig. 2 Distribution of known Carboniferous granites in Newfoundland. L. I. C., LaScie Intrusive Complex; C. B. P., Cape Brulé porphyry; L. B., Lockers Bay granite; S. L., St Lawrence granite.

orogen have been attributed to either irregularities in the geometry of the colliding continental plate margins¹³, or to an oblique collision¹⁴. The result, in both cases, is the formation of numerous small plates, wedges and splinters of continental crust, usually bounded by transcurrent faults. After the Hercynian orogeny the division of Newfoundland into a series of independent fault-bounded blocks could account for the distribution and origin of many of the geological features that are now observed in the Central Mobile Belt. High-angle faults, many of them transcurrent, abound in the central part of Newfoundland, and are especially numerous close to the boundary between the Central Mobile Belt and the Lomond Zone. Some time after collision, crustal thickening along with transcurrent faulting led to partial melting of the continental crust which produced Mid-Carboniferous magmatism and mineralisation. Deformed Carboniferous strata contained in a narrow, elongate, fault-bounded trough which trends from Cape Anguille in the south-west to White Bay in the north-east gives some stratigraphic significance to the interpretation of our isotopic data. The unconformable relationship of the Deer Lake Group with the basement and the Anguille beds in north-central Newfoundland testify to early and late Mississippian deformation¹⁵.

Both the stratigraphic and isotopic evidence support the model of Dewey and Burke¹, which includes southern Europe colliding with Europe/North America in Mid-Carboniferous times. The later post-Stephanian (Maritime or Alleghenian) disturbance further to the south coincided with Africa south of the South Atlas Fault sliding westward to collide with North America¹⁶.

For convenience, and to follow earlier workers, we use the term 'Hercynian Front' here, even though its meaning is poorly defined.

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Slow recolonisation of deep-sea sediment

USING the submarine ALVIN, experiments on deep-sea benthic communities have been designed to follow the recolonisation of defaunated deep-sea sediments and to provide estimates of growth rates and time to maturity in individual species. Small brood size and the high proportion of adults in a number of deep-sea benthic taxa suggest relatively low rates of recruitment, growth and mortality in the deep sea¹. Large individuals (8.4 mm) of the deep-sea bivalve, *Tindaria callistiformis*, have been estimated to be at least 100 yr old². Measurements of microbial activity and community respiration indicate rates one to three orders of magnitude lower than in shallow water^{3,4}. In contrast with the view that there is a general slowing of life processes, the first *in situ* deep-sea measurements on molluscan borers in wood panels have indicated rapid rates of recruitment, growth and maturation. Wood is, however, an ephemeral resource and its occurrence in the deep sea is unpredictable, while the majority of deep-sea habitats are thought to be highly predictable, therefore favouring less opportunistic life histories.

Studying community succession in defaunated sediments gives hope of predicting the rate of recovery of deep-sea communities following a disturbance and assessing the normal role of disturbance in structuring these remarkably diverse communities. The *in situ* placement of defaunated sediments in benthic studies is similar to the use of fouling panels or artificial three-dimensional habitats.

In September 1972, the research submersible ALVIN placed boxes (50×50×10 cm) of azoic sediment at a depth of 1760 m at the first Woods Hole Oceanographic Institution Permanent Bottom Station (PBS 1) (39°46'N, 70°40'W). The sediment was previously collected in the same area at approximately the same depth with an anchor dredge. It

Table 1 Results of experimental boxes of azoic sediment placed and recovered in shallow water and the deep sea

Station	Time exposed (months)	Density of individuals (m ⁻²)	No. of species	No. of individuals	Sample area (m ²)
Buzzards Bay Control (10 m)	—	48,375	61	1,935	0.040
Buzzards Bay Expt	2	35,714	47	704	0.021
PBS 1 Control (1,760 m)	—	5,189	> 103	454	0.0875
PBS 1 Expt 1	2	160	14	43	0.250
PBS 1 Expt 2a	26	564	31	141	0.250
PBS 1 Expt 2b	26	536	10	15	0.028

Table 2 Species contributing more than 2.3% of the individuals in the 2-month and 26-month experimental boxes and 25 control cores

25 Control cores		26-month box PBS 1		2-month box PBS 1 (1 individual is 2.3%)	
<i>Prochaetoderma</i> sp.	7.5%	Gnathiid isopod	13.5%	<i>Priapulus atlantisi</i>	30.2%
Spionid (undescribed)	5.1%	<i>Neopodarke woodsholea</i>	12.8%	<i>Xylophaga</i> sp.	23.3%
<i>Aricidea abbranchiata</i>	4.4%	<i>Capitella</i> sp.	11.4%	<i>Eulima stenostoma</i>	16.3%
<i>Glycera mimica</i>	4.2%	<i>Glycera mimica</i>	11.4%	<i>Prochaetoderma</i> sp.	4.7%
<i>Pholoe anoculata</i>	3.7%	<i>Nucula cancellata</i>	9.9%	Ophiuroid	4.7%
<i>Priapulus atlantisi</i>	3.3%	<i>Nucula cortica</i>	5.0%	<i>Xyloredo ingolfia</i>	2.3%
<i>Nucula cancellata</i>	3.1%	<i>Pholoe anoculata</i>	3.6%	<i>Dacrydium</i> sp.	2.3%
<i>Polycarpa delta</i>	2.9%	Ampharetid	3.6%	Pectenid bivalve	2.3%
<i>Tharyx</i> sp.	2.6%	Holothurian	2.8%	<i>Cocculina leptale</i>	2.3%
<i>Poecilochaetus fulgoris</i>	2.4%	<i>Polycarpa delta</i>	2.8%	<i>Dentalium</i> sp.	2.3%
				Sphaerodorid	2.3%
				Spionid	2.3%
				Epicarid isopod	2.3%
				Gnathiid isopod	2.3%

was kept at room temperature for at least 2 weeks, then frozen at -30°C for at least 1 week, and then thawed. One box was retrieved in 1974 after it had been on the bottom for 26 months and the second box was cored *in situ*, also after 26 months, with eight 35-cm² tubular cores. Similar tubular cores were also used in 1972 and 1973 to obtain a total of 25 control cores from surrounding sediments at PBS 1. A third box was placed at PBS 1 in July 1975 and retrieved after 2 months. Another box of the same size, containing similarly treated Buzzards Bay mud was placed on the bottom at a depth of 10 m in Buzzards Bay in July 1971 and was sampled after 2 months with six 35-cm² cores. All macrofaunal animals (excluding nematodes, podocypid ostracods and copepods) retained by 0.297-mm screens were used in the analysis. Subsamples from the experimental boxes indicated no spatial patchiness as was also true in the control cores from the sea floor even though the control cores were taken at several different times.

Experimental boxes showed an order of magnitude fewer individuals and species than surrounding sediments (Table 1). The 2-month box (PBS 1, experiment 1) was dominated by larvae of the wood-boring *Xylophaga* sp. and the loricate larvae of *Priapulus atlantisi*. The abundance of *Xylophaga* sp. probably results from the proximity of wood panels placed on the same dive. All individuals of the other taxa were post-larvae or juveniles except for two adult ectoparasitic snails (*Eulima stenostoma*) which may have crawled in or been carried in by an echinoderm passing over the box. The 26-month boxes (PBS 1, experiments 2a and 2b) were dominated by juveniles of four species and adults of an as yet undescribed capitellid polychaete of the *Capitella* species complex (Table 2). The finding of a species in the *Capitella* group in the deep sea is of considerable interest, since species of this group have been recognized as indicators of disturbed conditions in shallow-water environments⁷. Only a few individuals of the *Capitella* group have previously been found in extensive collections from the deep Atlantic⁸. Hartman found large numbers of worms of similar description from collections in the vicinity of freshwater aquifers off California at depths up to 500 m (ref. 9). Polychaetes, *Glycera mimica* and *Neopodarke woodsholea*, and a bivalve, *Nucula cancellata*, were among the five numerically dominant species. An ectoparasitic gnathiid isopod was the most abundant species in the box even though it is relatively rare in surrounding sediments. Of the 141 individuals in the box sampled completely, only 18 adults were found: 16 individuals of *Capitella* sp., a single individual of the capitellid polychaete genus, *Heteromastus*, and a single adult tunicate, *Polycarpa delta*. The smaller area sampled from the box cored *in situ* showed a similar species composition.

Three of the five most abundant species in the PBS 1 26-month boxes are predators or ectoparasites and 64%

of the polychaetes are in groups known to move about actively as adults; that is, most of the species settling in the boxes as larvae are relatively motile forms.

The results presented demonstrate a tremendous difference between rates of colonisation in shallow-water at a depth of 10 m and these rates in the deep sea. Comparison of two boxes sampled on the bottom (shallow-water and deep-sea) after 2 months indicates two orders of magnitude lower density in the deep-sea box. The 41 larval and post-larval individuals and two adults in the deep-sea box belong to 14 species. The shallow-water Buzzards Bay box contains thousands of individuals ranging from post-larvae to adults of at least 47 species. The difference is even more striking if the study on similar boxes placed in an intertidal mud flat is used as a basis for comparison⁷. The experiments in the intertidal zone demonstrate an even faster response with high densities of opportunistic species settling as larvae in the boxes and many growing to adults within a month. In the deep sea the densities do not rapidly increase to saturation levels. Indeed, more recent results from greater depths suggest even lower rates of colonisation. We cannot discount the possibility that densities are kept low by predation^{10,11}, but if this is so, the predation seems to be continuous, in that it is observed in both 2-month and 2-yr boxes, and does not result in patchy distribution within the boxes.

These results indicate that disturbance resulting in severe reduction of deep-sea fauna may be a long-lasting source of spatial heterogeneity (on the same scale as the disturbance) with the effects of disturbance apparent for at least 2 yr and probably much longer. Effects of disturbances 100 cm² or larger have not been observed in studies of patchiness in the deep sea, but the life and death of individual organisms can create local heterogeneity¹². Life processes as reflected in rates of colonisation and growth are remarkably slow in the deep sea. Thus, small-scale spatial and temporal mosaics resulting from disturbances on the scale of individual organisms may have a major role in structuring highly diverse deep-sea communities.

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5-¹²⁵I-iododeoxyuridine as prototype for radionuclide therapy with Auger emitters

MANY of the physical and biological requirements for treating cancer with internally emitting radionuclides are met by 5-¹²⁵I-iododeoxyuridine (¹²⁵IUdR). The decay of ¹²⁵I is associated with a highly localised deposition of energy¹. Cascades of very low energy and thus densely ionising Auger electrons are released. In addition, a charge transfer reaction takes place leaving the resultant tellurium nucleus with a transient mean net charge of +9 (ref. 2). These unique physical characteristics can be used for cell destruction by labelling DNA with ¹²⁵IUdR, a thymidine analogue. The marked radiotoxicity of ¹²⁵I incorporated into DNA as ¹²⁵IUdR has been demonstrated by lethality, mutations and DNA strand breaks in bacteria and bacteriophage³⁻⁷, by lethality and chromosome aberrations in mammalian cells cultured *in vitro*⁸⁻¹⁰, and by diminished survival of prelabelled tumour cells assayed *in vivo*¹¹. The intranuclear location is of major importance: ¹²⁵I located extracellularly⁹ or bound to cell membranes is much less toxic (unpublished work of R. L. Warters and K. G. Hofer). These findings suggested that ¹²⁵IUdR might have significant anti-neoplastic activity. We tested this hypothesis in a murine ascites tumour model^{12,13}. In a pilot study using therapeutic doses of ¹²⁵IUdR, the agent substantially augmented the median and absolute survival of treated animals. The critical factors affecting treatment of a 1-d-old ascites tumour model with ¹²⁵IUdR are detailed here.

The tumour used in these experiments arose spontaneously in the ovary of a C3H mouse¹⁴ and has been maintained as an ascites tumour by serial intraperitoneal (i.p.) transplantation in female C3HeB/FeJ mice (Jackson Laboratory). Median survival of untreated animals was determined after i.p. challenge with various tumour cell inocula (Fig. 1). The non-linearity observed at low tumour cell inocula (<10³ cells) suggests that host defenses may be effective in these circumstances. The relatively long survival of tumour-bearing mice facilitates quantitative evaluation of tumour cell killing after treatment with ¹²⁵IUdR and Fig. 1 can be used to calculate a cellular survival fraction. For example, the median survival of mice given 10⁷ cells is 19 d; if treatment prolongs it to 29 d, the treated animals died as though they had been given 10³ cells. This represents a survival fraction of 10⁻⁴. The calculation assumes that treatment with ¹²⁵IUdR does not alter the pathogenicity of

tumour cells destined to survive, and the absence of a shoulder on the *in vitro* survival curve of cells exposed to ¹²⁵IUdR supports this assumption^{9,10}.

Parameters of the cell cycle were determined for 1-d-old ascites tumour cells by the percentage labelled mitoses method¹⁵ (Fig. 2). From computer analysis of the data¹⁶, the total cell cycle time was calculated to be 14.3 h, DNA synthetic time 5.8 h, G₁ 6.3 h, G₂ 1.7 h, and mitosis 0.5 h. Because ¹²⁵IUdR is incorporated into DNA only during synthesis, it is important to match fractionation to the cell cycle. As the growth fraction for 1-d-old ascites cells approaches unity, all tumour cells can theoretically be labelled by appropriate schema. Optimal cell killing will be achieved by increasing the overall treatment time to cover several cell cycles and, in the absence of continuous infusion, maintaining an interval between treatments that is less than the DNA synthetic time.

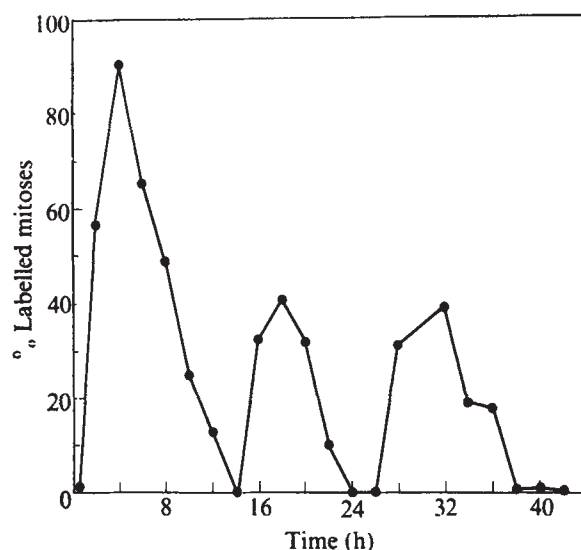


Fig. 2 Percentage labelled mitoses as a function of time after a single injection of 10 µCi ³H-thymidine administered 1 d after inoculation of 10⁶ tumour cells. Mice were killed at 2-h intervals; tumour cells were extracted from the peritoneal cavity by flushing, washed, fixed in methanol-acetic acid (3:1), and dropped on to slides. Autoradiography was performed with NTB-2 nuclear track emulsion (Eastman Kodak).

All experiments involved carrier-free ¹²⁵IUdR (New England Nuclear) administered i.p. starting 24 h after inoculation of 10⁶ tumour cells. The overall treatment time was 12–24 h with the interval between treatments being 4 h. Because ¹²⁵IUdR is dehalogenated rapidly *in vivo*¹⁷, potassium iodide was added to animal drinking water to block thyroid uptake of released radionuclide. Experiments were followed for at least 100 d. No gross acute or chronic toxicity was observed in treated animals and all deaths were associated with ascites formation.

Optimal conditions for ¹²⁵IUdR therapy were achieved by studying the effect of sequential treatments on tumour cell survival and establishing a dose-response relationship. In Fig. 3, tumour cell survival is plotted as a function of the number of treatments administered, the dose per treatment being constant. The effect of using four to seven sequential treatments is to extend the overall treatment time from 12 to 24 h, an increase approximating one cell cycle time. Because ascites cells are asynchronous, increasing the number of treatments and hence the overall treatment time would be expected to reduce further the survival fraction. The incremental reduction in survival fraction decreases with each successive treatment because fewer and fewer cells remain viable to incorporate ¹²⁵IUdR.

Tumour cell survival as a function of the dose of ¹²⁵IUdR per treatment is shown in Fig. 4. Seven treatments were

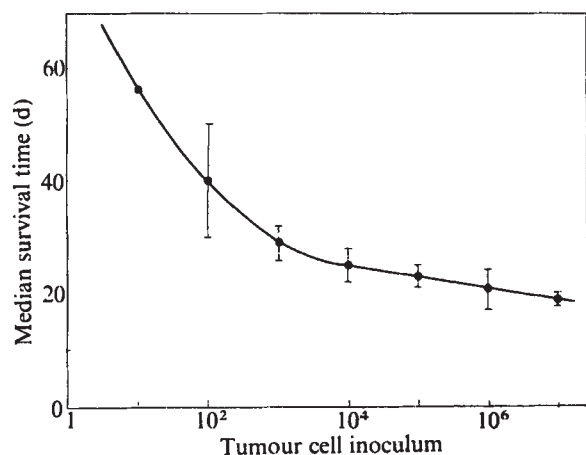


Fig. 1 Median survival $\pm$ standard deviation of untreated mice after i.p. injection of various tumour cell inocula. The median survival after injection of 10 cells represents the pooling of data from 14 experiments, in six of which fewer than half the animals died when followed for 150 d.

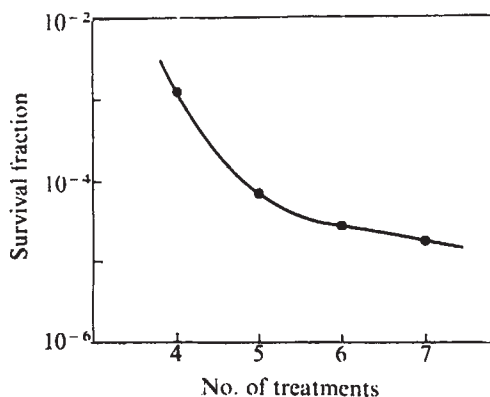


Fig. 3 Tumour cell survival as a function of sequential treatments with ^{125}I UdR (25 μCi every 4 h).

administered at 4-h intervals beginning 24 h after tumour cell inoculation. There is a steep and marked reduction in survival fraction at doses up to 15 μCi per treatment with a plateau observed at higher doses.

Both Figs 3 and 4 are complementary in that seven treatments of 25 μCi ^{125}I UdR result in a survival fraction of about 10^{-5} . Considering that the challenge dose was 10^6 cells, these data suggest that there is a very small fraction of tumour cells that are non-cycling or have long generation times. Increasing the number of treatments and overall treatment time or the use of continuous infusion might have killed these few remaining cells but perhaps at the risk of damaging critical normal tissue cell renewal systems.

In other studies^{12,13}, we showed that Na^{125}I , ^{131}I UdR and Na^{131}I had no anti-neoplastic activity when administered in similar doses and using a similar regimen to that used for ^{125}I UdR. The decay of ^{131}I is by β -minus emission and is not associated with any significant yield of Auger electrons or a charge transfer reaction. No anti-neoplastic activity was observed with non-radioactive UdR administered in amounts comparable with ^{125}I UdR. That the anti-neoplastic activity of ^{125}I UdR is primarily due to the radiotoxicity of ^{125}I incorporated into DNA is further supported by the

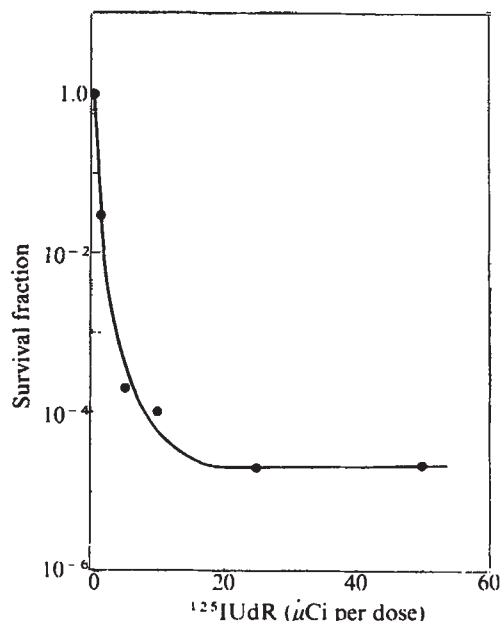


Fig. 4 Dose-response function for ^{125}I UdR therapy of 1-d-old ascites tumour cells. Seven treatments were given at 4-h intervals beginning 24 h after tumour cell injection.

failure to observe X-ray sensitisation in tissue culture at concentrations of UdR that are toxic when used as ^{125}I UdR⁹.

We conclude that ^{125}I UdR has marked anti-neoplastic activity and provides the basis for a new approach to the treatment of cancer. We appreciate that any early ascites tumour model is a propitious system in which to test the efficacy of cell cycle active agents. In particular, a favourable therapeutic ratio results from the direct access of drug to tumour cells in the peritoneal cavity before ^{125}I UdR enters the general circulation where dehalogenation occurs; such dehalogenation in turn protects normal cell renewal systems. Further work will be necessary to determine the efficacy of ^{125}I UdR in solid tumours where the growth fraction is considerably less than unity and drug access is less direct. Nevertheless, the exquisite radiotoxicity of ^{125}I incorporated into DNA represents a challenge to synthesise and evaluate other labelled agents with high nuclear affinity.

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First North American record of the extinct panda *Parailurus*

THE living pandas are restricted to the mountains of south-eastern Asia where both the giant panda (*Ailuropoda*) and the lesser panda (*Ailurus*) occur. They overlap in geographical range only in Szechuan, China, and there they are separated altitudinally, with the lesser panda favouring higher elevations¹. Pleistocene records extend the range of the giant panda into Burma and eastern China, but the lesser panda has apparently not had a significantly wider range during the Quaternary². Considerable controversy surrounds the phylogenetic relationships of both genera as they represent some of the most derived of terrestrial Carnivora. Recent marshalling of anatomical evidence on the giant panda has demonstrated its fundamental affinity with other members of the Ursidae³. The position of the lesser panda is equivocal although it is usually assigned to the Procyonidae. Procyonids are widely thought to be restricted to the New World, but recent work on various Oligocene and early Miocene forms in Europe⁴ and North America⁵ indicate that the procyonids had a Holarctic distribution at that time. Regardless of its broader phylogenetic affinity, the presence of medial and late Miocene relatives of the lesser panda in Pakistan and western Europe (*Sivanusua* Pilgrim, 1931=*Schlossericyon* Crusafont, 1959)⁶

and their absence in the New World makes an Old World origin for this group highly likely. Thus the discovery of a single tooth in North America almost identical to European specimens of the extinct lesser panda *Parailurus* is unexpected, and must be explained by immigration from Asia.

It has long been known that Eurasiatic mammalian immigrants had an increasingly important role in the late Cainozoic fauna of North America, and in Quaternary time such allochthonous groups came to dominate the large mammal fauna of the New World⁷. Recent study^{8,10} of Pliocene (Blancan) assemblages of the north-western United States has added new taxa to the growing list of forms first appearing in the New World during that interval. The discovery of remains of the extinct panda *Parailurus* Schlosser, 1899, in the Taunton Local Fauna of the Ringold Formation of Washington adds another element to this list. It is interesting that the Ringold and correlated Glens Ferry formations (see below) contain several mammals representative of the oriental temperate woodland fauna that appear simultaneously in Europe in the Pliocene. In the New World these immigrants include the grison *Trigonictis*⁸ (close to *Enhydriactis*), the bear *Ursus* (*U. abstrusus*⁸ close to *U. boeckhi*), true otters (*Lutrinae*, represented by *Satherium*⁸ in North America) and primitive Cervidae with antlers of the 'three-point plan' of Geist⁹ (*Bretzia*¹⁰ with palmate antlers similar to Eurasiatic late Miocene *Cervavitus*). The recent discovery of the petauristine squirrel *Cryptopterus* in the Blancan of Florida¹¹ adds another oriental element to the Pliocene fauna of North America. In European sites yielding *Parailurus*, similar associations of mammals occur in the late Pliocene at localities of late Ruscinian (Csarnotian) age in England (Red Crag)¹², West Germany (Wölfer sheim)¹³ and Rumania (Barót-Köpec)¹⁴ and those of early Villafranchian age in Italy (Arondelli¹⁵ of the Villafranchian stratotype area), and Czechoslovakia (Hajnáčka)^{16,17}. The known Pliocene faunas of Asia are of different character and seem to represent a steppe or savanna (mixed grassland and forest) biotope, that, judging from available records¹⁸, extended northwards to the latitude of Lake Baikal. There is evidence for a Pliocene temperate forest biome that ranged at even higher latitudes across Holarctica¹⁹, and thus a pathway is present to permit the widespread occurrence of the fauna associated with such environments.

The Taunton Local Fauna was obtained from flat-lying fluvial deposits resembling those of the stratotype Ringold Formation at White Bluffs on the Columbia River, 24 km to the south. The higher topographic position for the Taunton site suggests that it may equate with the uppermost part of the type Ringold sequence, above the occurrence of the White Bluffs Local Fauna¹⁰. Fish remains are the most abundant fossils at the Taunton Site and most of these pertain to a perch (*Archoplites taylori*) and a catfish (*Ictalurus vespertinus*), both of which are common in the Glens Ferry and type Ringold formations. Mammals are mostly known from isolated teeth. Most common are *Equus* (*Dolichohippus*) *simplicidens*, two species of the rabbit *Hypolagus*, and an antilocaprid similar in size to the living pronghorn. On the whole, the Taunton Local Fauna is similar to both the White Bluffs Local Fauna and the Hagerman Fauna. But the following differences are apparent when comparisons are made with the former: the presence of two species of *Hypolagus*, neither being the White Bluffs form; the presence of *Pliopotamys* cf. *minor* and the giant beaver *Procastoroides*; the presence of the badger *Taxidea*; the presence of *Canis lepophagus* rather than *C. davisii*; the absence of a more primitive *Equus* with short protocones; the rarity of cervids and the peccary *Platygonus*, and the presence of abundant antilocaprid remains. Some of these differences may be due to ecological bias. The stream which deposited the sediments containing

the Taunton Local Fauna seems to have been a relatively minor tributary. Riparian forests that probably broadly lined the major stream at White Bluffs may also have extended as a narrow belt into the Taunton tributary with savannah or open grassland nearby. This situation would explain the large percentage of rabbits, horses and antilocaprids as well as the presence of a smaller number of presumably forest animals at Taunton. Other differences can be construed as indicating closer temporal equivalence of the Taunton Local Fauna with the younger Hagerman than with the older White Bluffs Local Fauna. Such faunas are attributed to the earliest half of the Blancan Mammal Age of North America.

Biochronologically the occurrence of a species of *Parailurus* close to *P. anglicus* in North America serves to reinforce the correlation of the early Blancan faunas of which it is a part with those of Late Ruscinian and early Villafranchian Age in Europe²⁰. Radiometric calibration of these biochronological units in North America and Europe confirms this biological correlation: the Taunton Local Fauna is no younger than the Hagerman Fauna, the latter dated at 3.2–3.5 Myr (ref. 21). The early Villafranchian Etouaires Local Fauna of the Perrier Plateau, Auvergne, is just above a volcanic ash dated 3.4 Myr and is overlain by pumice dated 3.3 Myr (ref. 22). If the Viallette Local Fauna from the Auvergne is taken as Late Ruscinian in age and has a minimum date of 3.8 Myr (ref. 20) (established by the date on the overlying basalt), the Ruscinian–Villafranchian boundary would fall between 3.4 and 3.8 Myr. In round figures the known biochron of *Parailurus* would fall across this boundary probably within the interval 3.0–4.0 Myr.

The North American *Parailurus* is represented by a highly diagnostic upper right first molar (Los Angeles County Museum 10808) collected by George F. Beck of Yakima, Washington from fluvial gravels and cross-bedded sand (284.0 m above sealevel) that in turn overlies laminated chalky clay and are overlain by late Quaternary loess, 0.8 km east of Taunton station (LACM Locality 6408, 46°41'N latitude, 119°21'W longitude), Adams County, Washington. This tooth is longer anteroposteriorly than it

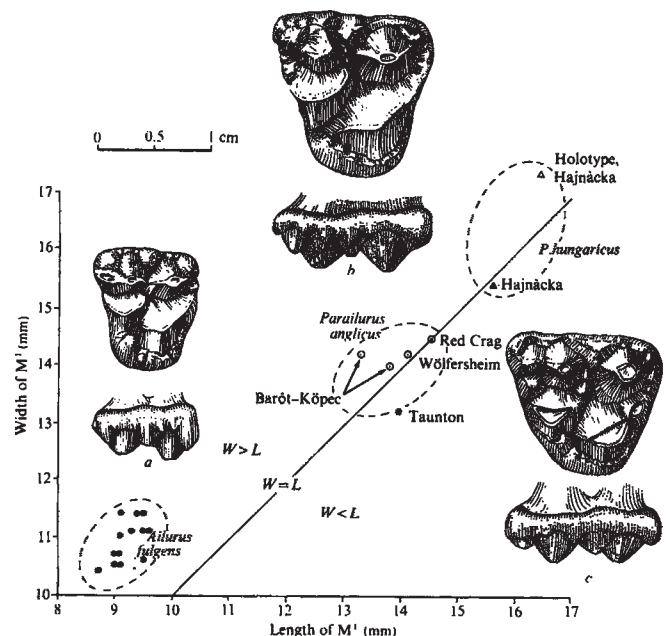


Fig. 1 Occlusal and labial views of right first upper molars of living and fossil lesser pandas, drawn to the same scale. a, *Ailurus fulgens* AMNH 80164, Recent, zoo; b, *Parailurus anglicus*, Senckenberg Mus. Wö 21, Pliocene, W. Germany; c, *Parailurus* sp., LACM 10808, Pliocene, Washington. Regression plot of length-width of the first upper molar.

is wide transversely (length 14.0 mm, width 13.2 mm) in contrast to European *Parailurus* in which the teeth are more nearly equidimensional or slightly wider than long. *Ailurus fulgens*, the living lesser panda, has smaller first molars that are markedly wider than long. The complex crown pattern of the Taunton form agrees in most details with those known for *Parailurus* as shown in Fig. 1, where LACM 10808 is compared with the specimen from Wölferstheim (Senckenberg Museum Wö 21), West Germany. The labial cingulum is weaker than in other *Parailurus* particularly that behind the mesostyle where stylar cusps form a more complex structure in *P. anglicus* and *P. hungaricus*. Likewise, the lingual cingulum is weaker in the American form giving the tooth a more triangular occlusal outline. But too few specimens of any population of *Parailurus* are known from which to judge the significance of the small differences that can be observed. The sample of *Ailurus fulgens* available (14 individuals from the American Museum of Natural History collection, three wild, the remainder zoo animals) shows a rather remarkable uniformity in size and morphology for an arctoid carnivore, suggesting that the difference in size between *P. anglicus* and *P. hungaricus* is probably significant biologically and that the morphological differences noted between the American and European forms are also significant. At present it seems most prudent to designate the American panda simply *Parailurus* sp., noting that among the described European species it compares most favourably with the late Ruscinian *P. anglicus*.

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Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors

THE colonisation of bacteria on human mucosal tissues is now recognised as an important step in the infectious process^{1,2}. To colonise mucosal surfaces, the organisms must first bind to the epithelial cells of these tissues, or they are discarded by the host's physico-anatomical defence mechanism (sneezing, coughing, urine flush and so on). The attachment of bacteria to epithelial cells can be demonstrated *in vitro*^{3,4} and is probably mediated by surface components of both types of cell⁵. It was found^{6,7} that lipoteichoic acid on the surface of group A streptococci is responsible for the attachment of these organisms to oral mucosal cells. For Gram negative organisms, pili mediate their binding to epithelial cells^{8–9}. In a study with *Proteus mirabilis* it was found¹⁰ that the presence of pili is important for the organisms to adhere to both oral and bladder mucosal cells as well as to initiate retrograde infection in experimental animals, probably by enabling the bacteria to adhere and to colonise pelvic epithelial cells¹¹. Nothing is known, however, about the putative receptor sites required for bacterial attachment on the mucosal cell membrane. We present here data indicating that attachment of *Escherichia coli* to epithelial cells is mediated by mannose (or mannose-like) receptors present on the surface of the latter.

In vitro adherence was studied by methods described previously⁴. Briefly, 0.5 ml of a suspension of epithelial cells (10^6 cells per ml in phosphate buffered saline, 0.1 M PO₄ and 0.15 M NaCl, pH 7.2–7.4) from the oral cavity of one of us (I.O.) was mixed with 0.5 ml of washed bacterial cells (*E. coli* strain 3092, a K₁₂ derivative¹², or strain B, adjusted to 5×10^9 organisms per ml, or group A type 12 streptococci, 2×10^8 organisms per ml). The mixture was rotated end over end for 30 min at room temperature, then non-adherent bacteria were separated from the epithelial cells by differential centrifugation. The pellet of epithelial cells obtained after four washings with water was resuspended in 0.05 ml phosphate-buffered saline and placed on a slide. After drying, the preparation was stained with gentian violet and examined using light-field microscopy. Adherence was recorded as percentage of epithelial cells containing 50 bacteria or more. When the K₁₂ or B strains of *E. coli* cells were mixed with a suspension of epithelial cells, the bacteria bound avidly to the cells (Fig. 1a) as shown previously for streptococci^{4,5}. Only *E. coli* cells collected in the late stationary phase or from cultures 24–72 h old, were attached to the epithelial cells.

D-Mannose and methyl α -D-mannopyranoside (α MM) inhibited the adherence to the epithelial cells of both strains of *E. coli* but not of streptococci (Table 1). None of the other sugars tested inhibited epithelial binding of these organisms. The inhibition by α MM of adherence was dose related and was linear in the range of 2–6 mg sugar per ml. In the presence of α MM (25 mg ml⁻¹) the *E. coli* cells did not adhere; they were separated from the epithelial cells and each type of cell was washed three times to remove the α MM. On remixing the cells, epithelial attachment of the bacteria was restored, indicating that the inhibition of adherence by the sugar is reversible and probably does not involve any irreversible alterations on either type of cell.

Our observations suggest that saturation of binding sites on the bacterial surface by a sugar prevents the attachment of these organisms to the epithelial membrane receptor, which contains mannose or a mannose-like structure. This conclusion is supported by the following results. (1)

Table 1 Inhibition of adherence of *E. coli* to epithelial cells by carbohydrates, lectins and oxidation with sodium metaperiodate

Inhibitor used*	Concentration in reaction mixture (mg ml ⁻¹)	% Adherence†
Methyl- α -D-mannopyranoside	25	5
	5.0	25
D-mannose	25	10
	5.0	30
D-galactose	25	99
N-acetyl-D-glucosamine‡	25	85
Wheat germ agglutinin	0.5	99
Peanut agglutinin	0.5	75
Concanavalin A	0.5	28
Oxidation of epithelial cells with sodium metaperiodate§		0

*The sugars or lectins^{13, 14} were incubated with suspensions of epithelial cells and bacteria for 30 min at room temperature, then treated as described in text.

†This was calculated as percentage adherence in the presence of sugars or lectins compared with control (without inhibitor) which was taken as 100% adherence.

‡The percentage adherence in the presence of other sugars (25 mg ml⁻¹) was in all cases > 90%. The sugars tested were: D-rhamnose, L-fucose, maltose, D-glucose, methyl- α -D-glucopyranoside, and methyl- α -D-galactopyranoside.

§An epithelial cell suspension (0.5 ml, 10⁶ ml⁻¹) was incubated with 10 mM of sodium metaperiodate for 10 min at room temperature. The cells were then washed twice with phosphate-buffered saline, resuspended in 0.5 ml of the same buffer, and mixed with *E. coli* suspension as described in the text.

Epithelial cells treated with sodium metaperiodate, a reagent known to cleave the C-C bond between vicinal hydroxyl groups of sugars, completely inhibited the binding of *E. coli* (Table 1) but did not affect binding of streptococci. (2) Concanavalin A, which is known to bind to D-mannose and D-glucose residues¹³, inhibited attachment to cell surfaces of *E. coli* but not of streptococci. The other lectins tested (wheat germ agglutinin—specific for N-acetyl-D-glucosamine (ref. 13), and peanut agglutinin—specific for D-galactose (ref. 14)) did not inhibit the binding of *E. coli* or of streptococci. (3) Both soluble yeast mannan and intact yeast cells, which are known to contain mannans on their cell surface¹⁵, agglutinated *E. coli* cells (Fig. 1b and c). Incubation of a suspension of *E. coli* with mannan (7 μ g ml⁻¹) or 10³ yeast cells per ml, gave visible agglutination of the bacteria after 30 min, characterised by a diffuse carpet of cells on the bottom of the tube. Agglutination of *E. coli* by both mannan and yeast cells (*Saccharomyces cerevisiae*) could be completely blocked by D-mannose or by α MM at a concentration of 25 mg ml⁻¹, but not by any other sugar tested at the same concentration.

Addition of D-mannose or α MM (25 mg ml⁻¹) to epithelial cells to which *E. coli* was preattached caused rapid (within 2–5 min) displacement of the organisms from the epithelial

cells. After washing the epithelial cells four times with a solution containing α MM (6 or 12 mg ml⁻¹), 55 and 85%, respectively, of the preattached bacteria were removed from the cells. Likewise, mannan (2 mg ml⁻¹) removed bacteria attached to the epithelial cells (Fig. 1b), but D-glucose, glycogen or D-galactose did not displace the bacteria.

Our results suggest that the binding of *E. coli* to epithelial cells is mediated by a mannose-specific, lectin-like substance present on the surface of *E. coli* which binds to a mannose-like receptor site on the mucosal cell. Indeed, extraction of *E. coli* K₁₂ cells (1 g wet weight) with phosphate-buffered saline, adjusted to pH 8.5 (1 ml) at room temperature, gave a solution which contained a lectin-like substance specific for mannose, as shown by the following findings: (1) The extract agglutinated yeast cells at a dilution of 1:20; (2) this agglutination was inhibited by D-mannose or α MM (3 mg ml⁻¹) but not by any of the other sugars listed in Table 1 (25 mg ml⁻¹); (3) the extract, at 1:80 dilution, inhibited attachment of *E. coli* to epithelial cells; (4) the latter inhibitory activity was abolished by adsorption with yeast cells; (5) the agglutinating activity of the extract was stable to 59 °C (30 min), but was destroyed at 70 °C (30 min), or by treatment with trypsin (0.3 mg ml⁻¹, 2 h, 37 °C). Further work on the purification and characterisation of the *E. coli* lectin-like substance is in progress.

Production of mannose-specific lectins by members of Gram negative organisms, including strains of *E. coli*, has been reported^{16–19}. In most of these studies the target host cell was the erythrocyte. Duguid *et al.*¹⁶ reported that mannose inhibited binding of *E. coli* to intestinal epithelial cells, but this was not investigated in any detail and was overlooked in subsequent studies². In agreement with the epithelial binding reported here, the bacterial haemagglutination was very weak in the logarithmic phase of growth and became strong only at 24–48 h growth¹⁹.

Our report provides evidence suggesting that the mannose-specific bacterial haemagglutinins^{16–19} enable the organisms to attach and colonise human mucosal tissues. Furthermore, mannose and its derivatives, which act as receptors for *E. coli* binding, can compete with the epithelial membrane-bound mannose to dissociate the organisms from the cell surfaces. Studies with concanavalin A suggest that mannose is widely distributed on the membrane of mammalian cells¹³. It would seem therefore that the normal distribution of Gram negative organisms on different mammalian mucosal tissues is not due to the presence or lack of the mannose receptor on epithelial cells. This is supported by the findings that, although the ileum rather than the jejunum of the gut is normally colonised by *E. coli*, the epithelial cells from both regions

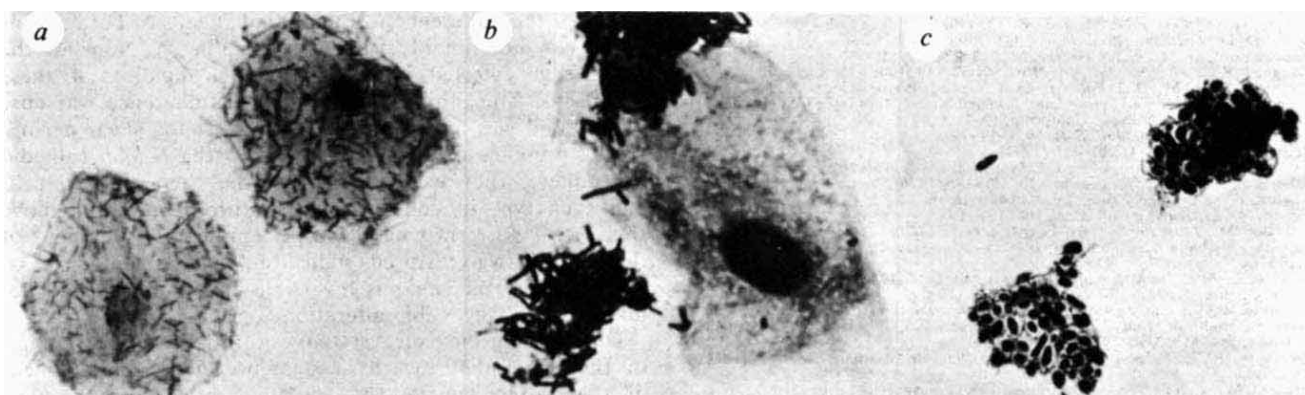


Fig. 1 Binding of *E. coli* to: a, human epithelial cells ($\times 400$); b, yeast mannan (Sigma) after removal from epithelial cells ($\times 1,000$); and c, yeast cells (*Saccharomyces cerevisiae*).

bound *E. coli* equally well²⁰. The mechanism of attachment of streptococci to epithelial cells is different from that of *E. coli*, for none of the sugars tested inhibited the lipoteichoic acid-mediated streptococcal attachment^{4,1}. In this regard it has been suggested that certain human tissues, such as sections of heart valves, lack receptors for *E. coli* but not for streptococci²¹.

In conclusion, our finding that mannose, a sugar found on most mammalian cell surfaces, acts as receptor for binding of *E. coli*, which is one of the most common commensals of man, may provide an approach to the elucidation of the mechanism of bacterial adherence and subsequent colonisation on mucosal tissues.

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Suppression by heparin of smooth muscle cell proliferation in injured arteries

INTIMAL smooth muscle cell (SMC) proliferation dominates the early phase of healing after arterial endothelial injury^{1–6}; what growth factors are responsible for this mitotic activity of an otherwise quiescent cell is not known. SMC growth *in vitro* is enhanced by platelet factors, insulin and lipoproteins^{7–11} and, according to some reports, can be diminished *in vivo* with various antiplatelet agents. Harker *et al.*⁸ have reported decreased myointimal thickening in homocystinaemic baboons treated with dipyridamole, and Friedman *et al.*¹¹ and Moore *et al.*¹² have reported suppression of SMC proliferation in the injured arteries of rabbits injected with anti-platelet serum. Intimal thickening was not diminished, however, in the injured carotid arteries of rats given various anti-platelet drugs¹³. Because the clotting system is inextricably linked to arterial injury and because thrombin is used to generate the platelet growth factor⁸ and may itself be a mitogen^{14,15},

we speculated that heparin might inhibit myointimal thickening. We report here the use of heparin to suppress intimal SMC hyperplasia in a rat model of arterial endothelial injury and suggest possible mechanisms for the heparin effect.

Endothelial injury was achieved by infusing air into isolated segments of right carotid arteries of 45 young male Sprague–Dawley rats (250–300 g, Charles River Breeding Laboratories) as described earlier^{1,2}. This procedure desiccated the endothelium. After blood flow was re-established and haemostasis obtained, the wound was irrigated with Betadine solution. Using a sterile technique, a silastic catheter was inserted into the left jugular vein and connected to an infusion system as described by Steiger *et al.*¹⁶. Initially all rats were infused with lactated Ringer's solution (Abbott) at 0.91 ml h⁻¹ using a syringe infusion pump. After 24 h, experimental rats were switched to an infusion of heparin (from pig intestinal mucosa, Elkins-Sinn). Control rats were infused continuously with Ringer's solution. Animals were killed at 5, 10 and 14 d by perfusion fixation^{1,2} after blood had been collected for clotting times¹⁷ and platelet counts. Carotid arteries were further fixed and processed as described earlier^{1,2} for light, scanning (SEM) and transmission electron microscopy (TEM).

Clotting times were prolonged in a predictable fashion; as noted before¹⁸, a linear relationship existed between the logarithm of the clotting time and the heparin dose. Heparin doses of 50 or 100 U kg⁻¹ h⁻¹ given intravenously yielded clotting times of 12.5 and 36 min (control 4.4 min). Platelet counts were not affected by heparin administration (control: $1.28 \pm 11 \times 10^6$ ($\pm$ s.e.); 50 U kg⁻¹ h⁻¹: $1.38 \pm 0.04 \times 10^6$; 100 U kg⁻¹ h⁻¹: $1.38 \pm 0.11 \times 10^6$).

In earlier studies on uncatheterised control animals^{1,2}, we showed that after carotid injury the desiccated endothelium rapidly sloughed and was supplanted by a carpet of platelets. Endothelial regeneration proceeded from normal endothelium at the ends of the injured segment and was complete by 14 d; between 5 and 14 d smooth muscle cells migrated through the internal elastic lamina and proliferated in the intima. The intimal thickness was maximal at 14 d.

The right carotid arteries of catheterised control animals at 5 d demonstrated partial endothelial regeneration and a dense monolayer of platelets in the residual central denuded portion of the injured segment. Unlike uncatheterised controls, at 10 and 14 d, endothelial regeneration in catheterised control carotids was still incomplete in the central zone; in place of endothelium and platelets, modified smooth muscle cells lined the luminal surface. A possible explanation for this failure of endothelial regeneration is that in stressed animals or after long arterial injuries^{3,13}, rapidly growing SMC can temporarily form a luminal surface and impede endothelial regeneration. At 10 d, all injured carotids showed marked intimal thickening due to SMC proliferation; at 14 d, the intimal thickening was even more pronounced. The injured right carotids of heparin-treated rats exhibited the same features as catheterised controls at 5 d with partial endothelial regeneration at the ends and a dense carpet of platelets in the middle of the injured segment; the platelets in heparin-treated rats were morphologically (SEM and TEM) similar to controls. At 10 d, a small central residual zone of denudation covered by platelets remained and at 14 d endothelial regeneration was complete as in uncatheterised controls. At 10 d, there was only slight evidence of intimal thickening which nevertheless was not significantly different from controls. But, at 14 d, because of increased intimal thickening in control carotids, the maximum ratio of intima to media (I/M) in carotids from heparin-treated rats was diminished significantly (Fig. 1 and Table 1). The

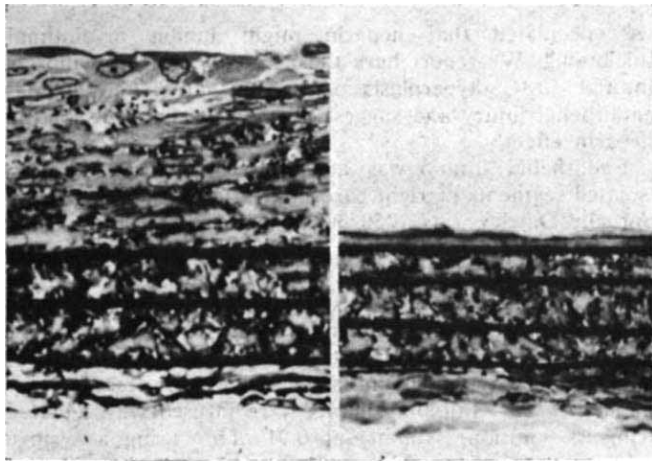


Fig. 1 Histological sections of injured right carotids in heparin-treated (right) and control (left) animals at 14 d. The maximum I/M ratio is 1.8 (control) and 0.2 (heparin). The minimal intimal thickening shown here in the right carotid of the heparin-treated rat is representative of findings in animals with a pronounced response to heparin; as can be seen in Table 1 there was considerable variation in intimal thickening under heparin treatment. TEM of these samples demonstrated only smooth muscle cells in the intima of the control carotid and a single layer of smooth muscle cells covered by endothelium in the intima of the carotid from the heparin-treated rat.

maximum I/M ratios for high and low dose heparin were similar and both were significantly different from control values. At most, one to three SMC layers were seen at this stage in the intima under the endothelium. The normal rate of endothelial regeneration in heparin-treated animals may have been related to the absence of luminal SMC.

These results indicate that heparin in doses sufficient to prolong clotting markedly suppressed intimal SMC proliferation after arterial endothelial injury even though endothelial regeneration (mitosis and migration) progressed unimpaired. Heparin did not seem to inhibit platelet-subendothelium interaction in the injured carotid.

This suppression of SMC proliferation may be the result of one or more effects of heparin because heparin is known to have many biological activities¹⁹⁻²¹. Heparin may act directly on the SMC^{19,21}. Alternatively, because of its charge, heparin may bind and inactivate platelet growth factors generated by platelet activity on the denuded arterial wall; this is particularly plausible in view of Ross's *in vitro* results demonstrating that the SMC platelet mitogen is a basic protein²². Heparin may achieve several effects indirectly through its action on the clotting system. Thrombin and the other serine proteases of the clotting system (XII, XI, IX, X and plasmin) are undoubtedly activated at the surface of the denuded segment and may accumulate in the arterial wall. One or more of these clotting factors may be mitogenic for SMC^{14,15}; thrombin, in particular, may generate mitogens from the adherent

platelets as suggested by *in vitro* work^{9,22}. Antithrombin III normally regulates the level of proteolytic activity by slowly inactivating the clotting proteases; this process is almost instantaneous in the presence of heparin²³. Thus, heparin through antithrombin III may affect the degradation of platelets and generation of platelet SMC growth factors by thrombin²⁴, and the direct activity of the clotting proteases on SMC. Arterial SMC proliferation may be just one example of the general phenomenon of wound healing suppressible by heparin; previous studies of wounds in skin, bowel, artery and bone indicate that heparin can prevent scarring if given in doses large enough to produce a continuous and significant state of anticoagulation²⁵⁻²⁸.

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***In vitro* culture and differentiation of normal mouse blastocysts**

WHEN preimplantation mouse embryos are transplanted to extrauterine sites, up to 20% develop almost normally to the egg cylinder stage (equivalent to about 6-7 d *in utero*). They then become disorganised and give rise to tumours, which are usually mature teratomas containing a wide range of differentiated tissues, but less frequently are progressively growing teratocarcinomas containing nests of undifferentiated pluripotent embryonal cells^{1,2}. There are many reasons why it would be interesting to reproduce such normal and abnormal development efficiently *in vitro*. Although a number of techniques have been used to culture preimplantation embryos, none of them has proved entirely satisfactory³⁻⁵; in the best conditions only 5-20% of 3.5-4-d blastocysts develop *in vitro* to form differentiated, foetal tissues. Most only give rise to extraembryonic endoderm and its associated mesoderm; these cell types proliferate extensively *in vitro*, and consequently most of the continuous lines derived from blastocyst cultures have probably originated from extraembryonic tissues⁶. In this paper

Table 1 Myointimal thickening at 14 d

	I/M
Control	1.9 ± 0.5 (5)
Heparin (50 U kg ⁻¹ h ⁻¹)	0.4 ± 0.2, <i>P</i> = 0.02 (5)
(100 U kg ⁻¹ h ⁻¹)	0.6 ± 0.1, <i>P</i> = 0.005 (10)
Total	0.5 ± 0.1, <i>P</i> = 0.0007 (15)

I/M ± s.e. was measured in right carotid arteries, 14 d after injury, which were cross sectioned at 500-μm stages along their entire length^{2,13}. Because the thickened intima at 14 d is almost entirely composed of smooth muscle cells¹⁻⁶ (Fig. 1), the maximum I/M ratio reflects the amount of SMC proliferation and is measured by using an ocular graticule by taking the ratio of the maximum intimal thickness (distance from the luminal surface to the internal elastic lamina) to the adjacent media thickness (distance from the internal elastic lamina to the most external elastic lamina). Numbers of rats in each group are shown in parentheses.

we describe a simple and reproducible method for culturing mouse blastocysts *in vitro* so that around 70% give rise to colonies which continue to grow for up to 4 weeks and produce a variety of differentiated tissues, including skin, nerve, beating muscle, cartilage and fibroblasts. The method relies on the technique of immunosurgery⁷ for killing and removing first the trophectoderm and then the hypoblast (primary endoderm) layers of the embryo, to yield clumps of isolated epiblast (embryonic ectoderm) (Fig. 1).

What subsequently happens to the epiblast cells depends entirely on the exact conditions in which they are grown. When they are incubated in bacteriological dishes so that they cannot adhere to the substratum they degenerate

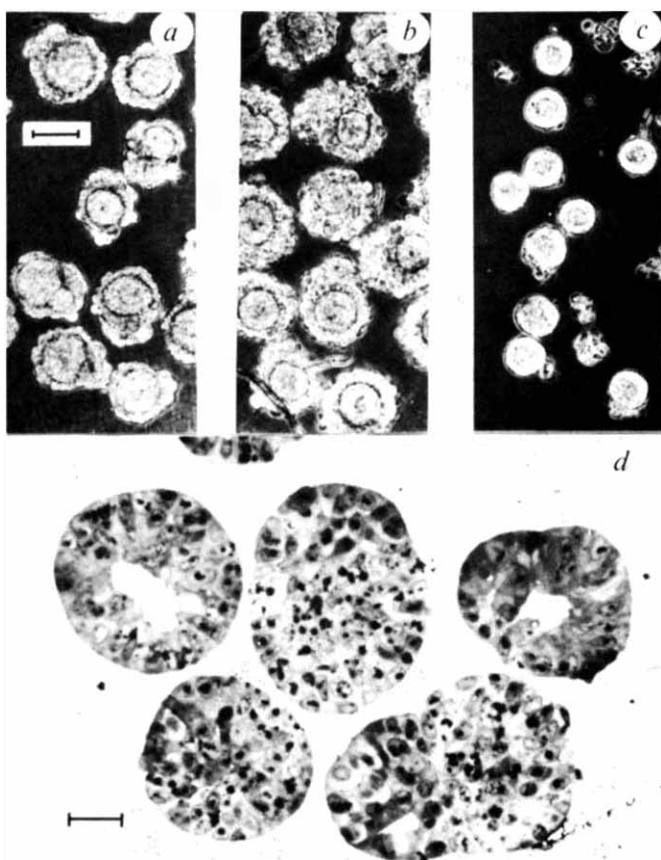


Fig. 1 Cultured embryos before and after removing the hypoblast layer by immunosurgery. Blastocysts are flushed from the uteri of C3H mice on day 4 of pregnancy and, after removal of the zona pellucidae with Pronase[®], are incubated overnight at 37 °C in a humidified atmosphere of 95% air/5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) with either 10% foetal calf serum or 20% human cord serum (HCS, heat inactivated for 30 min at 56 °C). Next day, the embryos are transferred to DMEM containing rabbit anti-C57BL mouse spleen antiserum⁷ diluted 1:50. After 30 min at 37 °C the embryos are washed twice in DMEM with 0.5% bovine serum albumin (BSA, Sigma fraction V) and transferred to a solution of guinea pig complement (Wellcome, 1:10 dilution) and incubated for 30 min at 37 °C, by which time all the outer trophectoderm cells have lysed. The embryos are washed once in DMEM plus 0.5% BSA and incubated in DMEM with 20% HCS. After 48 h all the inner cell masses have developed an outer layer of hypoblast cells and in some cases the inner epiblast cells have formed a hollow sphere (a). If the immunosurgery procedure is repeated the hypoblast cells are lysed (b) and can be removed by pipetting the embryos up and down in a finely drawn Pasteur pipette (c). Most of the isolated epiblast cell masses are solid, a few are hollow and a very few are asymmetric, with a cluster of larger, more densely staining cells to one side (d). Of 27 epiblast masses serially sectioned at 1 µm, all had an almost continuous outer layer of basement membrane material and the few hypoblast cells still attached were clearly dead. a, b and c, Phase-contrast microscopy (bar: 100 µm); d, isolated epiblast cells, fixed in 2.5% glutaraldehyde post-fixed in OsO₄ and embedded in Araldite. Sections were cut at 1 µm and stained with Toluidine blue (bar: 25 µm).

within 24 h. When incubated in tissue culture dishes, however, they rapidly adhere and spread out to form a sheet of cells surrounding a central clump (Fig. 2a); at 48 h some cell division is still occurring, but each colony finally degenerates by about day 4. The viability of these cultures cannot be improved by coating the dishes with gelatin.

When the stripped embryos are incubated in the presence of a monolayer of mouse feeder fibroblasts about 70%

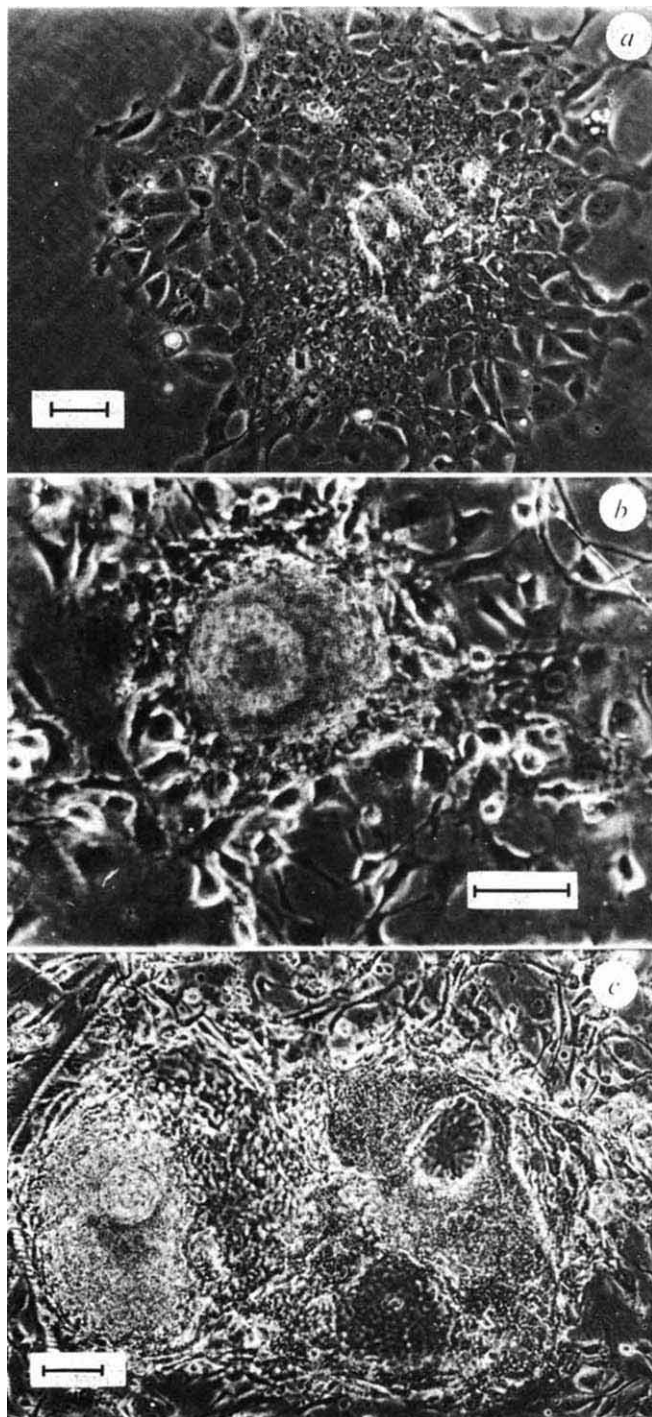


Fig. 2 Epiblast cells in culture. a, After incubation on a tissue culture dish (Nunc, Flacon or Lux) for 24 h. b, After 3 d on a layer of 2×10^5 feeder fibroblasts on a sheet of Melinex plastic placed in the bottom of a 35-mm tissue culture dish. c, After 3 d on a layer of 1×10^6 feeder fibroblasts on a 50-mm tissue culture dish. All cultures were incubated in DMEM containing 20% HCS. The mouse fibroblasts were treated before plating out with Mitomycin C ($10 \mu\text{g ml}^{-1}$) for 3.5 h so that they could not divide. In a typical experiment, 20–30 epiblast cell masses were added per dish and the medium changed every 2 d. Phase-contrast microscopy (bar: 100 µm).

of them survive for up to 4 weeks and form colonies containing a variety of differentiated tissues. Even in the presence of the feeder layer, however, the epiblast cells are sensitive to the underlying substrate; to facilitate electron microscope studies, the stripped embryos were sometimes grown on feeder layers on Melinex plastic (Boyden Data Papers, London, UK) and on this substratum they form rounded and compact colonies (Fig. 2*b*). When the epiblast cells are placed on feeder layers on standard tissue culture plastic, they form colonies which are much more spread out, grow more rapidly in a disorganised way and form a variety of differentiated tissues (Fig 2*c*).

Nineteen embryos which had been incubated on Melinex for 3 or 4 d were serially sectioned and examined in the light and electron microscope. The colonies fell into three main categories (Fig. 3). The simplest consists of a flattened vesicle of cuboidal or columnar cells, often embedded in the feeder layer (Fig. 3*a*). The second category contains a similar hollow vesicle of cells but, in some planes of sections,

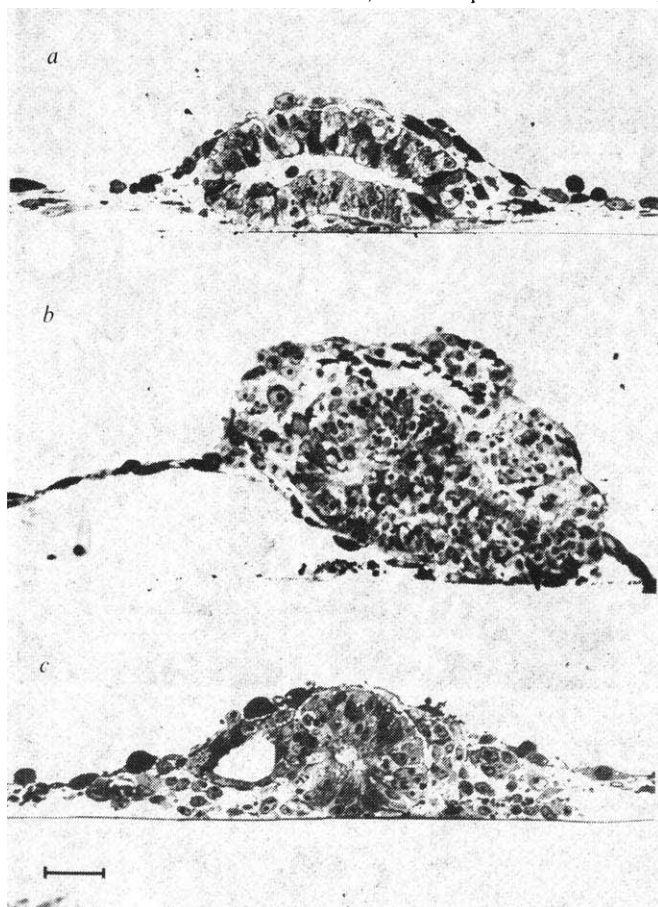


Fig. 3 Sections of epiblast cells grown on feeder fibroblasts on Melinex plastic, showing the three commonest forms of internal organisation. Epiblast cells were cultured for 3 d (*b*) or 4 d (*a* and *c*) and then fixed, sectioned and stained as described in Fig. 1*d*. In *a* and *c* the feeder fibroblasts form an almost continuous layer over the embryo; in *b* the embryo cells are on top of the feeder layer, which has partially detached from the Melinex (bar: 50 μ m).

this is seen to be disorganised and surrounded by clusters of other cells (Fig. 3*b*). The third category contains, in addition, a second vesicle lined by flatter cells which laterally interdigitate and have more endoplasmic reticulum and microvilli. Both vesicles are surrounded by a thin basement membrane. The cuboidal cell vesicle often contains masses of cellular debris, suggesting that it is initially formed by cell death. The flatter cell vesicle contains floccular material, suggesting that it is secretory. The cells surrounding these vesicles have few intracellular organelles and are either in tightly packed clumps, or are stellate and touch only at

limited areas, often through contact zones; the stellate cells closely resemble mesodermal cells of normal 8-day embryos¹⁰. At the surface of the colonies some of the cells are occasionally joined laterally by tight junctions and desmosomes to form an outer layer, but this does not resemble morphologically the primary endoderm layer formed when isolated inner cell masses are incubated *in vitro* (Fig. 1*a*).

Colonies growing on feeder layers on tissue culture dishes are more spread out and, during the first few days of culture, can be seen to contain closely packed cells which resemble undifferentiated teratocarcinoma cells. Patches of fibroblast-like cells appear in most colonies by 4–5 d, together with convoluted ridges and vesicles of epithelial cells. Beating muscle and neurones are seen in about 20% of the colonies after a week in culture. Later many colonies contain clumps of large, rounded cells and also flattened cells with very large nuclei and granular cytoplasm. By 3 weeks, extensive sheets of fibroblast and epithelial cells may be seen. Seven colonies were examined histologically at around this time; all contained simple and squamous epithelium overlying mesenchymal tissue. Keratin pearls were found in two (Fig. 4*a*) and muscle and cartilage in one (Fig. 4*b*).

The experiments described here establish that isolated epiblast cells are able to differentiate *in vitro* into a variety of tissues. It seems, however, that this only occurs if the cells retain some three dimensional association following their isolation because if they are allowed to spread out into a monolayer they do not differentiate (Fig. 2*a*). The function of the feeder layer may be to provide growth factors which allow epiblast cell division to continue fast enough to counteract cell migration. If the feeder cells and embryos are plated out into separate halves of the same dish, most

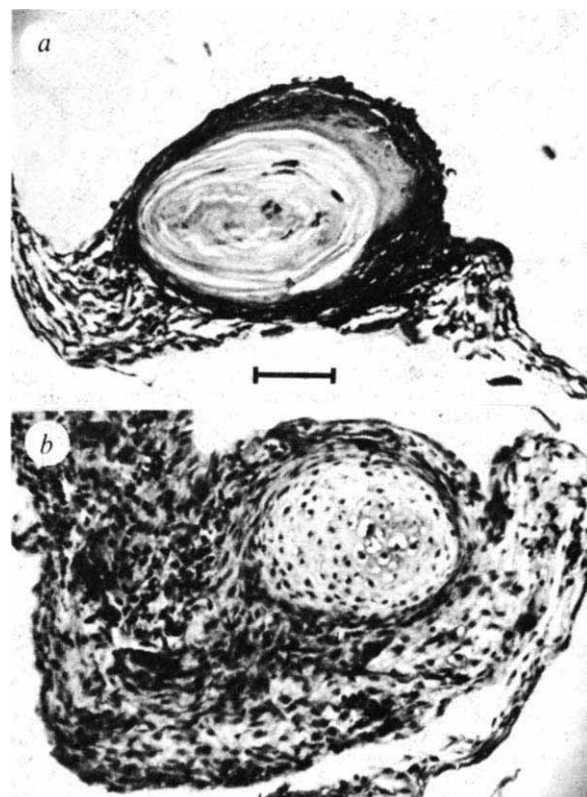


Fig. 4 Tissues present in colonies derived from epiblast cells incubated as described in Fig. 2*c*. Colonies were scraped off the dish, fixed in formal saline and embedded in wax. Sections were cut at 4 μ m and stained with haematoxylin and eosin. *a*, Section showing a pearl of keratinised squamous epithelium in a colony which had been growing for 22 d. *b*, Section showing cartilage and muscle in a colony which had been growing for 17 d (bar: 50 μ m).

of the epiblast cell masses still spread out and die, but a few (2/20) retain central clumps of cells and give rise to nerve, muscle, fibroblasts and skin.

The behaviour of isolated epiblast cells in culture provides several pieces of information about the way in which normal embryogenesis may be controlled. For example, the fact that mesoderm forms in the complete absence of a hypoblast layer shows that signals from the endoderm cells are not required for mesoderm differentiation. Similarly, because the epiblast cells do not reform an outer layer of primary endoderm, this suggests that either they are irreversibly committed to forming tissues other than endoderm, or they are not receiving the correct stimulus for this step under our culture conditions.

Several laboratories have studied the fate of embryonic ectoderm dissected manually from 8 d rat¹¹ or 6–7-d mouse^{12,13} embryos and transplanted into extrauterine sites. In these sites the cells differentiate into teratomas and teratocarcinomas containing ectodermal and mesodermal tissues, as well as definitive endoderm tissues such as gut. This suggests that the endoderm of the mammalian foetus, like that of the chick¹⁴, is being derived from cells of the epiblast and not from the hypoblast. So far we have not positively identified tissues of the definitive endoderm in our cultures, but it is possible that the formation of two vesicles in some colonies (Fig 3c) represents an attempt by the cells to make definitive endoderm.

We are now using the method to isolate lines of early embryonic cells, including undifferentiated pluripotent cells equivalent to the embryonal cells of teratocarcinomas. The method may also provide a way of deriving cell lines from parthenogenetic embryos and from embryos of mice with mutations at the *T* locus.

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Parathormone-stimulated resorption of devitalised bone by cultured osteoclast-type bone cells

WE recently reported the isolation from mouse calvaria of two different bone cell populations that express unique changes in metabolism when exposed in culture to parathormone and calcitonin^{1,2}. In one cell type, termed *CT*, parathormone increased the synthesis of hyaluronate and the activity of acid phosphatase. In the second cell type, termed *PT*, parathormone inhibited citrate decarboxylation and decreased the activities of prolyl hydroxylase and alkaline phosphatase. Calcitonin blocked the effects of parathormone in the *CT* cells but not in the *PT* cells. Since the changes elicited in the separate popu-

lations by parathormone closely resembled those exhibited by calvaria maintained in organ culture⁴, it seemed probable that the isolated cells retained unique properties in culture that are characteristic of their function in the intact tissue. On the basis of these results the *CT* cells were provisionally identified as osteoclasts and the *PT* cells as osteoblasts³. The major physiological result of the action of parathormone on bone *in vivo* or in culture is resorption of the calcified extracellular matrix, so we evaluated this function in the isolated bone cells. Should either the *CT* or *PT* cells promote resorption *in vitro*, this would further demonstrate that they retain differentiated function in culture and would aid in establishing their identity. We describe here a procedure which allowed us to test for resorptive properties of our isolated bone cell populations. We found that parathormone markedly stimulated the capacity of *CT*, but not *PT*, cells to resorb a calcified bone matrix substrate—a property in keeping with the identification of the *CT* cells as osteoclasts.

CT bone cells (populations 1 and 2) and *PT* bone cells (populations 4 and 5) were isolated from calvaria of 2–3-d-old mice and were maintained in primary culture for 7 d as described before³. They were then resuspended at 2×10^6 cells per ml in BGJ_b medium⁵ (Gibco), containing 5% foetal calf serum. Devitalised calcified matrix substrates were prepared from the calvaria of 3-d-old mice. After removal of extraneous tissue the calvaria were frozen at -20°C for 3–5 d. Before use they were thawed at room temperature, then refrozen and thawed three times. In some experiments the starting calvaria were prelabelled *in vivo* with ^3H -proline and ^3H -hydroxyproline by obtaining them from pups of mothers that were injected with 250 μCi of $[2,3\text{-}^3\text{H}]$ proline 1–2 d before delivery. These substrates were shown to be devitalised by their inability to resorb in culture conditions or to incorporate exogenous ^3H -thymidine and ^3H -leucine. The calvarial substrates were placed in individual wells of multi-well culture dishes (Linbro) and maintained in 5% CO_2 in air at 37°C for 1 h. A 0.05-ml aliquot of the bone cell suspension (10^5 cells) was deposited in the concavity of each calvarium. Medium without cells served as a control. The cells and matrix were then incubated for 2 h (5% CO_2 in air, 37°C) to facilitate cell adherence, after which they were covered with 1.0 ml of fresh BGJ_b medium alone or containing parathormone 200 ng ml^{-1} (2×10^{-8} M) or calcitonin 110 ng ml^{-1} (3×10^{-8} M), or both. In some experiments 2.5×10^5 d.p.m. of $^{45}\text{CaCl}_2$ at an initial specific radioactivity of 1.6×10^5 d.p.m. μmol^{-1} were included in this addition. The initial concentrations of calcium (Ca) and inorganic phosphate (Pi) in the incubation medium were 1.6 and 1.0 mM, respectively, and the pH was 7.4.

Culture was continued for 72 h. Histological examinations (our unpublished data) showed that the cells remained viable and formed a compact layer closely apposed to the surface of the bone matrix. Occasionally large multinucleated cells were seen in both populations, but more often in the *CT* layers. The medium was separated from the bone and analysed for Ca and Pi (ref. 6). ^{45}Ca and pH (at 37°C , gas phase 5% CO_2 in air). To determine ^3H -proline and ^3H -hydroxyproline, samples of the media were hydrolysed for 24 h with 6 N HCl and samples of the hydrolysate were dansylated and chromatographed on thin-layer polyamide sheets⁷ with carrier dansyl-proline and dansyl-hydroxyproline. The proline and hydroxyproline spots were cut out, eluted with 0.5 ml of 50% pyridine and radioactivity was assayed. The cells affixed to the calvarial matrix were tested after 72 h of culture for their capacity to synthesise ^3H -hyaluronate from $[6\text{-}^3\text{H}]\text{glucosamine}$ and to decarboxylate $[6\text{-}^{14}\text{C}]\text{citrate}$, as described previously³. All assays of radioactivity were performed by liquid scintillation spectrometry⁸.

Figure 1 summarises the results of growing bone cells on the bone matrix substrate for 72 h. With either the *CT* or *PT* cell populations in contact with the matrix there was release of Ca and Pi into the medium. In cultures of *CT* cells, however, there was substantially more ^3H -hydroxyproline release from

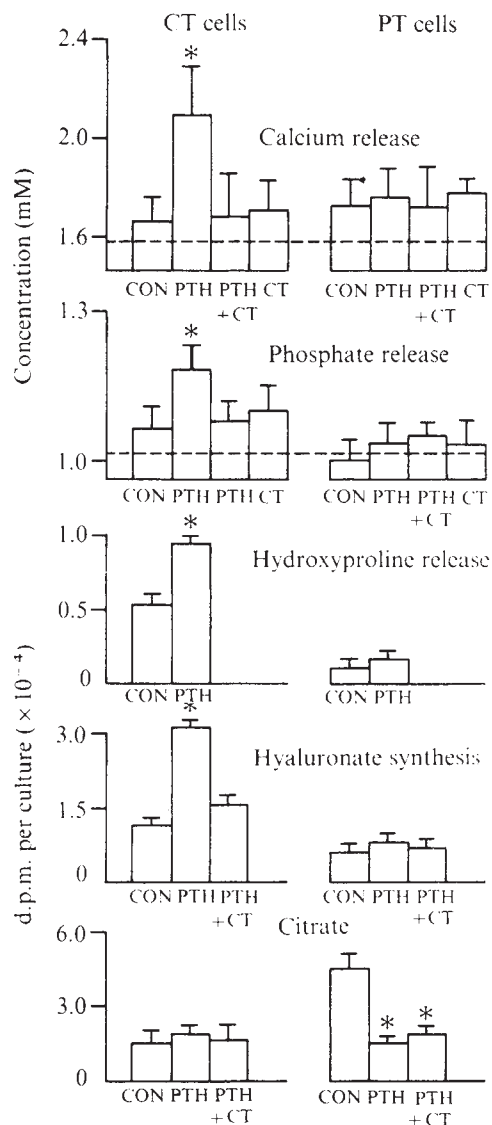


Fig. 1 Differential effects of parathormone and calcitonin on bone resorption by osteoclast-type (CT) and osteoblast-type (PT) isolated bone cell populations. CT and PT cells were isolated and subcultured (10^5 cells per culture) on devitalised bone matrix for 72 h as described in the text. CON, no additions to the medium; PTH, parathormone 2×10^{-8} M; CT, calcitonin 3×10^{-8} M; PTH + CT, both hormones. Values are shown as means $\pm$ s.e.m. of three to four cultures. Baseline values shown for calcium, phosphate and hydroxyproline are those obtained when devitalised calvaria were cultured for 72 h without cells. Dashed lines denote the initial concentrations of calcium and phosphate in the BGJ₆ culture medium. * = significant difference from CON, $P < 0.05$ (Student's *t* test).

prelabelled bone matrix. This indicates that in CT cultures resorption of collagenous matrix accompanied the dissolution of bone mineral to a greater extent than in PT cultures. This difference in resorption could not be correlated with differences in acidification of the medium by the cells (final pH in all cultures was approximately 7.2) or with differences in the uptake of ^{45}Ca by the matrix (final specific radioactivity in all cultures was approximately 0.9 d.p.m. μmol^{-1}). In cultures of matrix incubated without cells, there was a net transfer of Ca and Pi from the medium to the matrix. The pH did not change and no ^3H -hydroxyproline was released to the medium.

Parathormone markedly stimulated the resorption of Ca and Pi by the CT cells and calcitonin blocked this action (Fig. 1). The hormone also stimulated the release of hydroxyproline. These changes in resorption were not accompanied by any alteration in pH of the medium or uptake of ^{45}Ca . In contrast, parathormone or calcitonin alone or in combination exerted no

significant effect on resorption of Ca and Pi by the PT cells. Figure 1 also shows that parathormone exerted its previously observed characteristic effects on the CT and PT cell populations³. That is, it stimulated hyaluronate synthesis in the CT cells but not PT cells and calcitonin blocked this action. Parathormone inhibited citrate decarboxylation in the PT cells but not in the CT cells and calcitonin did not block this effect.

To establish that the action of parathormone was specific, we tested unrelated peptides and H_2O_2 -oxidised (that is, biologically inactive) parathormone⁹. In three experiments, ACTH (1 U ml^{-1}), human somatotrophic hormone (1 μg ml^{-1}), angiotensin II (1 μg ml^{-1}), and oxidised parathormone (1 μg ml^{-1}) were added to CT cells in culture with devitalised matrix as described above. Native parathormone was the control. In each experiment these peptides failed to elicit removal of calcium from the matrix by the CT cells.

These observations reinforce our earlier conclusion³ that CT cells exhibit the characteristics of osteoclasts. The findings that the resorption of devitalised matrix by CT cells, but not by the PT cells, was stimulated by parathormone and that calcitonin inhibited this resorption are in keeping with what would be expected for osteoclasts acting *in situ*^{10,11}. Our results also show that when both cell types are in contact with matrix there is a small amount of resorption in the absence of exogenous parathormone. The substantially larger release of hydroxyproline from the matrix by the CT cells, however, indicates that even in these basal conditions the CT cells function in a manner more analogous to osteoclasts *in vivo*. The basis for demineralisation of the matrix by the PT cells is not as clear. It could be an inherent property of this cell type since osteocytes (probably derived from osteoblasts¹²) have been suspected of actively resorbing mineral¹³. Our culture technique facilitates characterisation of the individual isolated cell populations in contact with the physiological matrix in varying conditions. This approach may permit precise characterisation of the molecular processes of bone development and turnover and its control by a variety of hormonal and nutritional factors.

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T-cell-mediated cytotoxicity against herpes simplex virus-infected target cells

THE control of herpes simplex virus (HSV) infection by immunological mechanisms seems to be complex and is poorly understood. Neutralising antibodies to HSV plus complement seem to have no effect on the propagation of HSV infection, because HSV spreads to adjacent cells by passing through intercellular bridges¹⁻³. Anti-HSV antibodies plus complement, however, destroy virus-infected cells, but cannot prevent the spread of HSV, suggesting that the virus must be transferred to neighbouring cells before immune lysis occurs^{1,4}. Therefore if lymphocyte-mediated cytolytic mechanisms are instrumental in blocking the

spread of HSV *in vivo*, they ought to destroy infected cells at a very early stage in the viral maturation cycle, before infectious viral particles are produced. Indeed, effective antibody dependent cell-mediated cytotoxicity (ADCC) has been demonstrated *in vitro* using target cells infected with HSV^{1,2}. In this system K-cell-mediated destruction of virus-infected cells takes place very early in the infectious cycle³. As *in vivo* protection against HSV infection is predominantly T-cell-dependent^{4,5}, the *in vivo* spread of HSV infections may be controlled by virus-specific cytotoxic T lymphocytes (CTL). But no virus-specific CTL so far have been detected in the spleen of HSV-infected mice. Using a different experimental approach we have now shown that T lymphocytes of mice undergoing a local HSV infection, when transferred *in vitro*, differentiate into effective CTL. The CTL generated are highly cytotoxic for HSV-infected target cells.

HSV type 1 strain Lennette was propagated in primary rabbit kidney cells as described before⁶. About $1-5 \times 10^5$ plaque-forming units (PFU) of HSV were injected into the hind footpad of CBA/J mice. After 3 d the draining lymph nodes (LN) were isolated and teased, and single-cell suspensions were prepared. The cells were cultured for a further 72 h at 37°C, a period required to facilitate full differentiation of the sensitised CTL precursors (K. P. *et al.*, to be published). Thereafter, the LN cells were tested for the presence of virus-specific CTL in a 6-h cytotoxicity assay⁹ using thioglycollate-induced ⁵¹Cr-labelled, HSV-infected CBA macrophages as targets. The syngeneic target cells were infected *in vitro* with 2-5 PFU of HSV per cell 16 h before the ⁵¹Cr assay.

Representative results obtained in 17 experiments of this type are shown in Table 1. Accordingly, LN cells draining the site of a local HSV infection *in vivo*, when transferred *in vitro*, differentiated into cytotoxic lymphocytes (CL) with strong lytic activity towards HSV-infected syngeneic macrophages. In contrast, LN cells draining a site which had been

Table 2 Effect of anti-Thy 1.2 serum plus complement

Treatment of cytotoxic lymphocytes	% Lysis of HSV-infected CBA macrophages	
	40:1*	4:1
None	66	31
Complement alone	70	40
AKR anti-Thy 1.2 serum plus complement	6	3
AKR-serum plus complement	67	38

Cytotoxic lymphocytes towards HSV-infected targets were raised as described in Table 1. The cells were divided into four groups; one group remained without treatment, the others were treated either with complement, anti-Thy 1.2 serum plus complement or with normal AKR serum plus complement. The remaining cells were tested in a 4.5-h ⁵¹Cr assay for cytotoxicity towards HSV-infected CBA macrophages (2×10^4) at a cell dilution resulting in the ratio of CL to targets given (for the untreated group). Background lysis of the targets was less than 27%.

*Ratio of CL to target cells.

showed only marginal cytotoxic activity. On the other hand, LN cells draining a local HSV infection until day 10, on transfer *in vitro*, generated effective HSV immune CTL.

In four of 17 experiments the CTL generated were almost equally cytotoxic for non-virus-infected, normal syngeneic macrophage targets as for virus-infected cells (see experiment 172, Table 1). This result *a priori* would contradict a possible virus specificity of the CTL generated. In most experiments, however, the CTL generated lysed non-infected syngeneic targets only to a limited extent (experiments 165 and 179, Table 1), so the possibility of the existence of HSV-specific CTL was investigated further.

Because in the above experiments the CTL were assayed for cytotoxic activity in a 6-h ⁵¹Cr assay, and because HSV surface antigen was expressed on infected macrophages as early as 2-3 h after infection (K. P. *et al.*, to be published),

Table 1 Lysis of HSV-infected syngeneic macrophages by HSV-induced cytotoxic effector cells

Experiment no.	<i>In vivo</i> sensitisation	% Lysis of ⁵¹ Cr-labelled macrophages			
		50:1*	5:1	50:1	5:1
165	HSV	77	31	11	2
	Culture medium	-2	1	-3	-1
179	HSV	66	26	22	4
	Culture medium	-4	-1	0	0
172	HSV	46	21	42	20
	Culture medium	-1	0	0	-2

CBA mice received either a given concentration of Lennette strain of type 1 HSV virus in Dulbecco's modified Eagle's medium (DMEM) or DMEM alone by injection in the hind footpad. After 3 d the draining LNs were dissected, single-cell suspensions were prepared and the cells were cultured for additional 72 h in DMEM supplement with 5% foetal calf serum (FCS). The LN cells were then tested for cytotoxicity at various ratios of attacker cells to target cells against ⁵¹Cr-labelled syngeneic macrophages⁹. The thioglycollate-induced CBA macrophages were either HSV-infected or non-infected. HSV-infected macrophages were prepared by exposing the cells to Lennette strain type 1 HSV at a multiplicity of infection of 2-5 PFU per cell at 37°C in DMEM plus 5% FCS for 1 h at 37°C. The infected macrophages were washed and incubated for additional 15 h at 37°C. Assay time of the ⁵¹Cr cytotoxicity test (using 2×10^4 target cells) was 5-6 h. Background lysis of the infected or non-infected macrophages was less than 31%. The percentage lysis data were calculated as before⁹. Of a total of 17 experiments performed, three representative experiments are detailed.

*Ratio of attacker cells to target cells.

injected only with culture medium (control) did not develop cytolytic activity. Pretreatment of CL with anti-Thy 1.2 serum plus complement abrogated their cytotoxic activity (Table 2), strongly suggesting that the CL were T cells. LN cells not removed 3 d after local HSV infection and cultured for 3 d *in vitro*, but obtained *in vivo* 6 d after infection,

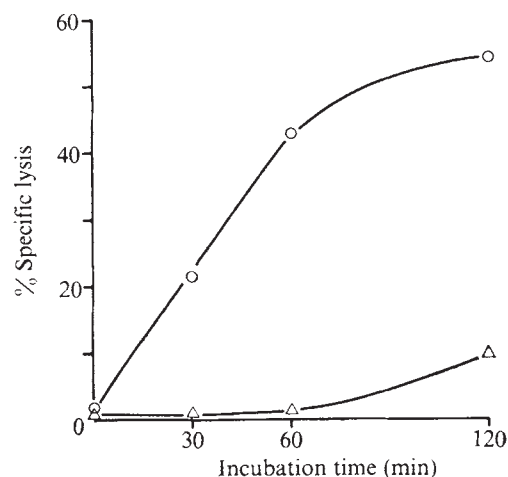


Fig. 1 Specificity of the CTL as assayed in a short-timed ⁵¹Cr assay. The assay depends on the fact that CTL can be inactivated by incubation at 45°C for 10 min, thus stopping the cytotoxic action of CTL on target cells at any given time. Further incubation for 3 h will lead to the manifestation of the previously set cytotoxic action (for details see ref. 10). CBA mice were injected with $1-5 \times 10^5$ PFU HSV into the hind footpad. Three days later the cells of the draining LN were prepared, cultured for additional 72 h *in vitro* and then tested for cytotoxicity towards HSV-infected¹ (○—○) or non-infected² (△—△) target cells (2×10^4). Replicates of mixtures of CTL and target cells were set up at a ratio of 50:1 and incubated at 37°C. At each of the time intervals indicated, triplicates of the two experimental groups were transferred for 10 min to 45°C, and then incubated for 3 h at 37°C. Percentage specific lysis was calculated as before⁹. For control 1, cytotoxic activity of CTL to HSV-infected macrophages at 37°C over 4 h was 62%. For control 2, cytotoxic activity of inactivated CTL (10 min at 45°C) at 37°C over 4 h was 2%. Background lysis of the targets was less than 28%. Similar results were obtained in five independent experiments.

it is possible that an infection of 'normal' macrophage targets may take place during the 6-h ^{51}Cr assay. Infectious HSV particles carried over during the ^{51}Cr test from the effector cell population to the 'normal' macrophage targets would thus induce an intra-experimental HSV infection of normal targets, and render them susceptible to the cytolytic action of the CTL generated. We therefore tested for virus-specific cytotoxicity in an experimental system in which the time of contact between CTL and target cells could be reduced to less than 1 h. In such assay conditions only targets which had been infected before the onset of the ^{51}Cr assay would be lysed.

The T-cell-mediated cytolytic effector phase can be dissected experimentally into a T-cell-dependent short-timed phase of antigen recognition and lethal hit, and into a T-cell-independent time-consuming phase of cytolysis¹⁰. It is therefore experimentally possible to reduce the time of interaction between CTL and the respective target cells to less than 1 h¹⁰. We used this approach in the HSV-system. The results (Fig. 1) clearly indicate that a contact time between CTL and HSV-infected targets of less than 1 h was sufficient for strong lysis of the targets. More important, the cytolytic activity of the CTL was restricted to HSV-infected targets. In these conditions, no lysis of normal targets was detected. Further studies showed that the lytic activity of HSV-specific CTL generated with the methodology described is restricted to recognition of HSV antigens in the context of syngeneic histocompatibility antigens (H-2 restriction).

T lymphocytes of mice draining a local site infected with HSV therefore differentiate *in vitro* into CTL. In a short-timed cytotoxicity assay these CTL are cytolytic for HSV-infected syngeneic targets. Non-infected syngeneic targets are not lysed. *In vitro* HSV-infected syngeneic targets are effectively destroyed by HSV-induced CTL, so such cells may be involved in controlling the *in vivo* spread of HSV infection. Because HSV-induced CTL lyse syngeneic targets as early as 2–3 h after infection and because HSV replication in infected cells requires at least 8–12 (ref. 5) HSV-induced CTL may be cytotoxic towards infected targets well before viral progeny is produced.

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Insulin inhibition of antibody-dependent cytotoxicity and insulin receptors in macrophages

INSULIN receptors have been demonstrated on various human white blood cells including monocytes^{1,2}, granulocytes³ and cultured lymphoblastoid cells⁴. Of these, the insulin receptor of the monocyte, a circulating macrophage, has been the most useful in the study of human disease^{5–9}, although insulin has no known effect on macrophage function. We demonstrate here that cultured mouse macrophages¹⁰ and macrophage-containing cell populations from normal mouse spleens both possess specific receptors for insulin with properties indistinguishable from those of the human monocyte, and that insulin, at physiological concentrations, inhibits the ability of these cell populations to lyse antibody-coated red cells (antibody-dependent cell-mediated cytotoxicity or ADCC).

^{125}I -insulin binding to cultured tumour macrophages (P388D₁ cell line), mouse spleen cells and human mononuclear cells demonstrated properties which characterise classical insulin receptors^{11–12}. The specificity of receptor binding was virtually identical for the three cell types (Fig. 1). Competition curves for ^{125}I -insulin binding by unlabelled porcine insulin revealed 50% inhibition of tracer

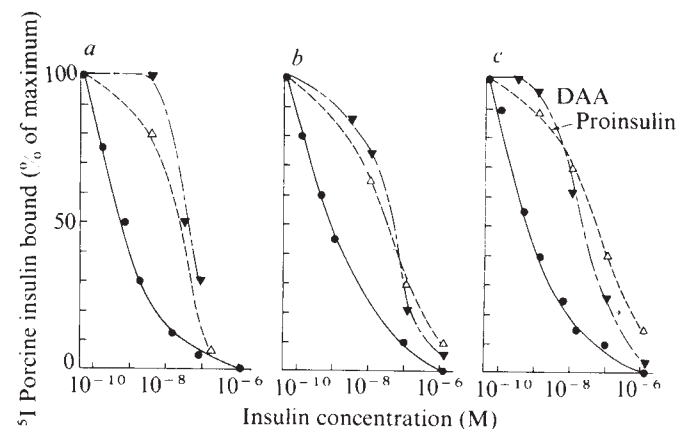


Fig. 1 Inhibition of ^{125}I -insulin binding by insulin analogues in *a*, human mononuclear cells, *b*, mouse spleen cells and *c*, cultured P388D₁ macrophages. Human mononuclear cells were prepared using Ficoll-Hypaque gradients by Boyum's method²⁰. The preparations contained approximately 20% monocytes by the criteria of latex bead ingestion, and 80% lymphocytes. Spleen cells were prepared from 6–10-week-old, DBA/2 male mice by a modification of the method of Mishell and Dutton²¹. P388D₁ cells were maintained in Eagle's MEM supplemented with 10% foetal calf serum with fresh culture medium added every 48–72 h and were used for ^{125}I -insulin binding studies approximately 24 h after fresh medium was added. Crystalline porcine insulin (Eli Lilly) was labelled with ^{125}I at a specific activity of 130–230 $\mu\text{Ci } \mu\text{g}^{-1}$ by a modification of the Hunter and Greenwood method^{22–23}. Tracer amounts of ^{125}I -porcine insulin (0.2 ng ml^{-1}) were incubated with 4×10^6 macrophages in 0.5 ml buffer (100 mM HEPES, pH 7.8 for P388D₁ and spleen cells or pH 8.0 for human monocytes) and increasing amounts of either unlabelled porcine insulin (●), unlabelled porcine proinsulin (△), or unlabelled DAA-insulin (▲). After 3 h of incubation (at 15 °C for P388D₁ and spleen cells or 22 °C for human monocytes), replicate 200- μl samples were transferred to microfuge tubes and centrifuged for 1 min. The resulting supernatant fluid was aspirated, the cell pellet was excised and the cell-associated radioactivity was counted. Nonspecific binding (radioactivity bound to cells in the presence of porcine insulin, $100 \mu\text{g ml}^{-1}$) was subtracted from total binding to yield specific binding. Maximum specific binding (100% value) for the human monocytes was 6%, for the mouse spleen macrophages 8% and for the cultured macrophages 13%.

binding at 10^{-8} M for all three cell populations. Porcine proinsulin and desalanine desasparagine insulin (DAA-insulin), which have 1–5% of the biological activity of porcine insulin, were 1–5% as potent as porcine insulin in competing for 125 I-insulin binding to the insulin receptors of these cells (Fig. 1). Thus, the receptors on all three cell types showed similar inhibition of insulin binding by insulin analogues with the receptor potency of the insulin analogue proportional to its biological activity.

In addition to specificity of binding, several physico-chemical properties of the insulin receptors in these three cell populations were similar to more classical insulin receptors. 125 I-insulin binding showed a sharp optimum at pH 7.8–8.0. The amount of 125 I-insulin bound in steady-state conditions increased with temperature in the order 4, 15, 22 and 37 °C. Both the association rate and the dissociation rate of 125 I-insulin binding increased with temperature, with the increase in the dissociation rate being greater than the increase in the association rate. Equilibrium analyses of insulin binding to the three populations of cells gave curvilinear Scatchard plots, and kinetic studies demonstrated that excess unlabelled insulin accelerated the dissociation of 125 I-insulin that had been bound previously to receptor. These latter two findings indicate site-site interactions among insulin receptors of the negatively cooperative type¹³.

Previous studies have shown that macrophages may act as effector cells in *in vitro*, antibody-dependent cytotoxicity assays^{14–16}. We found that insulin inhibited ADCC in both the tumour macrophages (P388D₁ cell line) and the mixed cell population prepared from mouse spleens (Fig. 2a, Table 1). Inhibition by insulin was most pronounced at low effector(macrophage)-to-target cell ratios (Fig. 2b, Table 1). At an effector-to-target cell ratio of 1.25:1, insulin at 10^{-8} M inhibited P388D₁-induced lysis by more than 70% and 10^{-10} M insulin inhibited lysis by 25%. With the mouse spleen cells, at ratios of 2.5:1, 10^{-8} M insulin inhibited lysis by 29% and 10^{-10} M insulin inhibited lysis by 15%. In P388D₁ cells, the inhibition of ADCC by insulin was dose-related for a wide range of insulin concentrations (Fig. 3). In both systems, insulin concentrations as low as 10^{-10} M, which is clearly within the physiological range, produced a significant decrease in cell lysis. This inhibitory effect of

insulin was reproducible in several experiments, the data in Figs 2 and 3 and Table 1 being representative.

In P388D₁ cells the inhibitory effect of insulin was exerted through the macrophages and not through insulin-induced alterations in target red cells. When the P388D₁ cells were preincubated with insulin and then washed before exposure to target cells, the effect of insulin on ADCC was still observed. If the target cells were first preincubated with insulin, thoroughly washed and then exposed to P388D₁ cells, however, 51 Cr release was not inhibited and was similar to that obtained in the absence of insulin (data not shown).

Both the mouse spleen cells and human mononuclear cells represent mixed cell populations. In the cell population prepared from mouse spleens, both insulin binding and cytotoxic activity seem to be due to the macrophages. When these spleen cells were depleted of macrophages by passage through a Sephadex G-10 column¹⁷, insulin binding was decreased by 75%, and ADCC was abolished (Table 1, depleted fraction). Moreover, when macrophage-enriched cells were eluted from the column before use, both cytotoxic activity and 125 I-insulin binding were restored (Table 1, enriched fraction). We previously found similar 125 I-insulin-binding results in enrichment and depletion experiments with the mixed mononuclear cell preparations from human blood; coupled with radioautography, these studies established that monocytes and not lymphocytes account for the observed insulin binding in this mixed cell preparation².

Macrophages from normal mouse spleens and cultured tumour macrophages (P388D₁ cell line) thus possess insulin receptors indistinguishable from those of the circulating human monocyte and other mammalian tissues. Furthermore, physiological concentrations of insulin alter a specific immunological function, antibody-dependent cell-mediated cytotoxicity, in both the spleen and tumour cells. In the spleen cells, insulin maximally inhibited ADCC by 30%, while in the more homogenous P388D₁ cells, insulin inhibited ADCC by more than 70%. The potency of insulin in the two cell systems may differ because spleen cells are a mixed cell population; multiple effector cells could exist in this preparation, with insulin acting on only a minority of the effector cells.

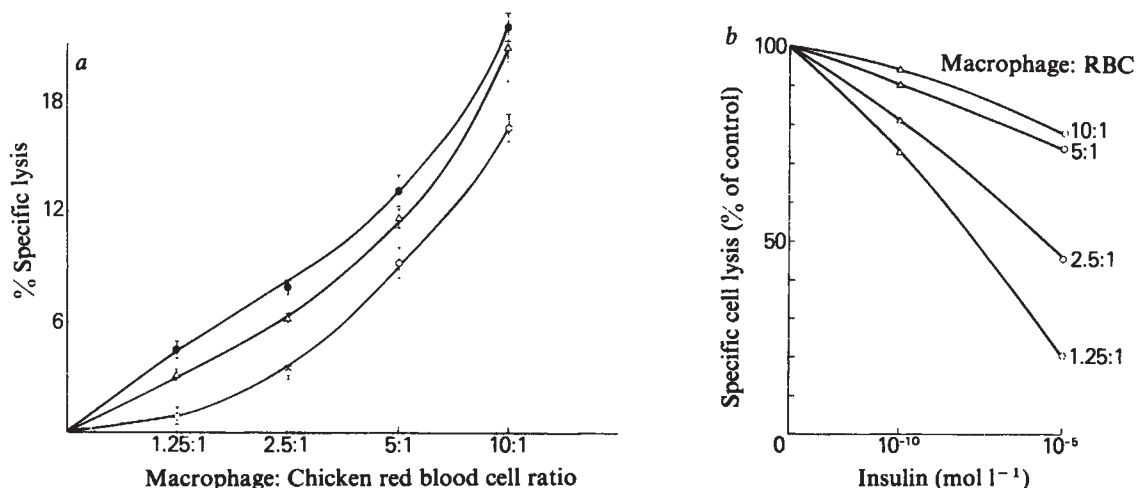


Fig. 2a. The effect of insulin on antibody-dependent cytotoxicity in cultured tumour macrophages (P388D₁ cell line). Chicken red blood cells were labelled with 51 Cr and coated with anti-trinitrophenyl antibodies as previously described²⁵. The effector cells (P388D₁ cells) were preincubated with or without insulin at 37 °C for 30 min. Various concentrations of effector cells were then added to 5×10^4 target red cells in a final volume of 1 ml, and incubated at 37 °C for 2 h. At the end of the incubation period the reaction mixture was centrifuged at 700g for 10 min at 4 °C. The resulting supernatant was counted and specific cell lysis was calculated using the formula: percentage specific lysis $(E - S)/(T - S) \cdot 100$, where E = counts of experimental group, S = counts from supernatant fluid of assay tubes containing only 51 Cr-labelled red cells (spontaneous release) and T = total radioactivity in assay tube. Spontaneous release in this experiment was 1.1%. Details of this ADCC assay have been previously described^{25,26}. The percentage specific lysis by effector cells preincubated with buffer alone ($\bullet$), with 10^{-8} M insulin ($\circ$) and with 10^{-10} M insulin (Δ) are plotted for different ratios of effector:target cells. All points represent the mean $\pm$ s.e. of triplicate samples. b. Inhibition of antibody-dependent cytotoxicity by insulin plotted as a function of effector-target cell ratios, which varied from 10:1 to 1.25:1. Lysis, calculated as percentage of buffer control at comparable effector-target cell ratio, is plotted as a function of insulin concentration.

Table 1 ADCC and ^{125}I -insulin binding in spleen cells from normal DBA/2 mice

ADCC by mouse spleen cells		[Insulin] (M)	% Specific lysis†	% Inhibition of lysis‡	P
E:T*					
10:1	—	—	45.9 ± 0.5		
10:1	10 ⁻¹⁰	10 ⁻¹⁰	43.9 ± 0.9	4.3	< 0.05
10:1	10 ⁻⁶	10 ⁻⁶	41.9 ± 0.8	8.7	< 0.01
5:1	—	—	21.9 ± 1.6		
5:1	10 ⁻¹⁰	10 ⁻¹⁰	18.6 ± 0.6	15.0	< 0.05
5:1	10 ⁻⁶	10 ⁻⁶	15.5 ± 1.1	29.2	< 0.025
2.5:1	—	—	9.9 ± 0.4		
2.5:1	10 ⁻¹⁰	10 ⁻¹⁰	8.3 ± 0.4	16.1	< 0.025
2.5:1	10 ⁻⁶	10 ⁻⁶	7.6 ± 0.2	23.2	< 0.025

Effect of macrophage enrichment and depletion on ADCC				
	E:T	% Specific lysis	% Control	% ^{125}I -insulin binding per 30 × 10 ⁶ cells
Initial cell preparation	5:1	20.0 ± 0.3	100	4.1
	2.5:1	10.2 ± 0.3	100	
Depleted preparation	5:1	0.9 ± 0.1	4.5	1.0
	2.5:1	0.6 ± 0.1	6.0	
Enriched (adherent preparation)	5:1	21.1 ± 0.5	106	6.8
	2.5:1	10.7 ± 0.2	104	

* Various concentrations of DBA/2 spleen cells (E) incubated with 2×10^5 target cells (T).

† Specific lysis calculated as described in Fig. 2. Spontaneous release in this assay was 7.3%.

‡ Calculated as percentage of control tubes without insulin.

The mechanism by which insulin alters macrophage-mediated ADCC is unknown. Rhodes has shown that insulin, at high concentrations (5×10^{-5} M) and over relatively long periods (72 h), decreases the number of F_c receptors on peritoneal macrophages¹⁸. The initial step in ADCC involves recognition by the macrophage F_c receptors of the F_c portion of the antibody which coats the target cell, so alterations of macrophage F_c receptors could decrease cell lysis. Whether insulin, at lower concentrations and over the shorter course of our experiments, could decrease the number or affinity of macrophage F_c receptors is unknown.

Strom¹⁹ has shown that physiological concentrations of insulin increase the cytotoxic potential of 'killer' T cells in a direct, lymphocyte-mediated cytotoxicity assay. Taken

together with our findings, these observations suggest that insulin can alter the immune system at multiple levels. The *in vivo* significance of our findings, and the potential role of insulin in immune function, however, remain speculative. We suggest that the cell populations described in this report offer unique model systems for studying the interrelations between insulin receptors and an insulin-mediated biological effect on immune function.

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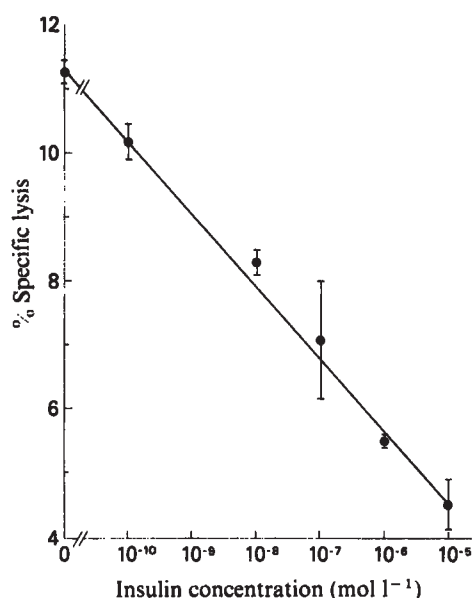


Fig. 3 Dose-response effect of insulin on antibody-dependent cytotoxicity in the cultured tumour macrophage. P388D₁ tumour cells were preincubated with various concentrations of insulin then assayed at an effector (macrophage)-target cell ratio of 5:1. Details of the assay are described in the legend to Fig. 2. Each point represents the mean ± s.e. of triplicate samples.

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Effect of flavone inhibitors of transport ATPases on histamine secretion from rat mast cells

INTERACTION of multivalent ligands with receptors (immunoglobulin E, IgE) on the surface of the mast cell stimulates the calcium-dependent degranulation and secretion of histamine^{1,2}. Secretion is also initiated by application of the calcium carrier substance (ionophore) A23187 (ref. 3), and so the effective common stimulus to this cell (and many others) is the entry of Ca^{2+} ions into the cytosol. The mechanism of the normal ligand-induced calcium entry process is not well understood⁴. Certain flavones, notably quercetin, interfere with the activity of membrane transport adenosine triphosphatases (ATPases) including the calcium-dependent ATPase which is associated with exclusion of calcium from the cytosol of cells. They are not simple ATPase inhibitors but probably act by increasing the efficiency of ion translocation, thus reducing the consumption of ATP (refs 5, 6). Also, the flavones (2-phenyl cromones) have some structural resemblance to the widely used anti-allergic drug cromoglycate whose activity may be expressed at the calcium entry pathway. We examine here the effect of these substances on histamine secretion from mast cells.

The effects of six flavones on antigen-induced histamine secretion are shown in Table 1. While two of these have little effect even at 100 μM , fisetin, quercetin, myricetin and kaempferol inhibit strongly at 33 μM . Fisetin, quercetin and myricetin interfere with transport ATPases while neither of the non-inhibitory flavones is active in this respect⁸.

Since ionophore-mediated release bypasses the normal calcium entry pathway regulated by multivalent ligands³,

it distinguishes between inhibitors which act on or before calcium entry and those which affect a later stage in the secretory process^{4,10}. Thus, whereas quercetin inhibits release stimulated by antigen and concanavalin A, which bind to IgE (refs 1, 9) (Fig. 1), release induced by ionophore A23187 is only slightly affected. It is unlikely that this latter effect arises from an action of the quercetin on the cells because (1) mixtures of A23187 and quercetin form visible precipitates; (2) the fluorescence emission spectrum of A23187 is red shifted in the presence of quercetin; (3) the permeability of liposomes rendered permeable to Ca^{2+} and Fe^{2+} with A23187 is reduced by the addition of quercetin.

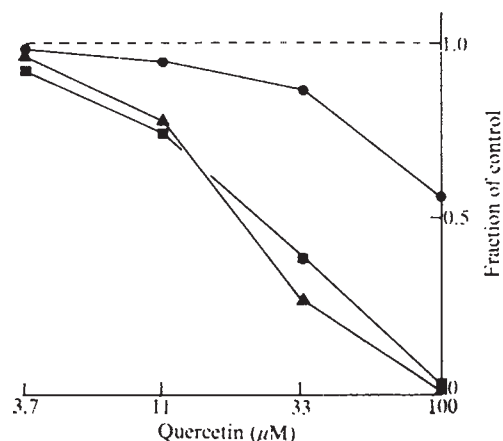


Fig. 1 Effect of quercetin on histamine release from mast cells stimulated with releasing agents. Methods are as in the legend to Table 1 except that the ionophore A23187, which is fluorescent, was extracted into hexane before histamine assay. Results are expressed as a fraction of control obtained in the absence of flavone. Mean s.e. = 0.04. ●, A23187 (5 μM) seven experiments; ■, antigen (10 $\mu\text{g ml}^{-1}$ egg white protein) seven experiments; ▲, concanavalin A (10 $\mu\text{g ml}^{-1}$) five experiments.

Our results support the idea that the effect of quercetin on ligand-stimulated cells involves blockade of the normal calcium entry pathway. A similar argument explains the action of the phosphodiesterase inhibitors theophylline and dibutyl cyclic AMP on mast cell secretion⁷, and dibutyl cyclic AMP was shown to prevent concanavalin-A-induced $^{45}\text{Ca}^{2+}$ uptake by T lymphocytes¹¹. Inhibition of mast cell secretion by cromoglycate could be mediated by a similar mechanism¹². Quercetin has negligible activity as an inhibitor of the cyclic AMP phosphodiesterase of human lung (J. Hare and I. F. Skidmore, personal communication) and unlike theophylline and dibutyl cyclic AMP the effect of quercetin is manifest when it is added to the cells simultaneously with the releasing ligand. In this respect, it resembles cromoglycate, but, unlike quercetin, the effect of cromoglycate is of short duration¹³, and is much less active on cells treated with phosphatidyl serine.

Our results indicate that we are dealing with a novel regulator of the calcium pathway.

At the concentrations used to inhibit mast cell secretion the activity of the flavones seems to be restricted to membrane transport ATPases^{14,15}. The effective flavones alter the activity of several ATPases including plasma membrane ($\text{Na}^+ + \text{K}^+$) ATPase of heart muscle¹⁶, mitochondrial ATPase¹⁵ and the Ca^{2+} ATPase of chloroplasts^{17,18}. The effect of quercetin on histamine release is unlikely to be due to inhibition of mitochondrial ATPase, as this should affect secretion induced by A23187 as well. It is also unlikely to be due to inhibition of the ($\text{Na}^+ + \text{K}^+$) ATPase since ouabain even at 1 mM had no effect on the antigen-induced process. The secretory event involves Ca^{2+} movements, so it is most likely that the effect of quercetin on the abolition of secretion from ligand-stimulated cells is directed at the Ca^{2+} ATPase in the plasma membrane.

Table 1 Effect of flavones (at 33 and 100 μM) on antigen-induced histamine secretion from rat mast cells and on Ca^{2+} -dependent ATPase activity of rabbit skeletal muscle sarcoplasmic reticulum

	Histamine secretion		Ca^{2+} -dependent ATPase activity	
	33 μM	100 μM	33 μM	100 μM
Fisetin	0.31	0.00	0.91	0.57
Kaempferol	0.59	0.24	0.64	0.30
Morin	0.99	0.82	0.95	0.74
Quercetin	0.37	0.00	0.69	0.37
Myricetin	0.42	0.26	0.74	0.37
Rutin	1.00	0.75	0.99	0.91

Results are expressed as a fraction of the control value obtained in the absence of flavone. Peritoneal mast cells purified to >80% homogeneity²⁵ were obtained from rats sensitised to egg white protein. Cells were preincubated at 37 for 5 min in Tyrode's solution containing 10 μM phosphatidyl serine before addition of the releasing agent and flavones. (The phosphatidyl serine was added to enhance the degree of ligand mediated secretion²⁶). After 2 min the reaction was ended by addition of ice-cold saline, and histamine in the supernatant was measured fluorimetrically²⁷. Any interference due to the flavones was compensated for by using appropriate blanks and standards. The Ca^{2+} -ATPase activity of rabbit muscle sarcoplasmic reticulum²⁸ was determined by measurement of the rate of H^+ release during ATP hydrolysis in a solution containing 250 mM sucrose, 100 mM KCl, 5 mM MgCl_2 , 8.2 mM potassium oxalate, 15 mM sodium citrate, 2 mM ATP, 10 mM Tris buffer. The pH was adjusted to 7.5 and free calcium concentration was brought to 10 μM by addition of 0.4 mM CaCl_2 . The reaction was initiated by addition of sarcoplasmic reticulum membranes, giving a final concentration of about 0.12 mg protein per ml. The specific activity of the preparation was about 1 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ at room temperature. Less than 10% activity was obtained in the absence of calcium.

We therefore tested the flavones as inhibitors of rabbit sarcoplasmic reticulum Ca^{2+} ATPase (Table 1). It would be desirable to compare the inhibition of the mast cell secretory reaction with Ca^{2+} ATPase from the same source, but as it has been claimed¹⁸ that the major Ca^{2+} -ATPase of the mast cell is reactive towards externally supplied ATP it is doubtful whether this enzyme is involved in Ca^{2+} transport. Even the association of this enzyme with the mast cell is open to doubt since the ecto-ATPase of 'purified' mast cell preparations is largely accounted for by the presence of contaminating macrophages²⁰. We have therefore preferred to work with the Ca^{2+} -ATPase of sarcoplasmic reticulum which has a well-defined Ca^{2+} transport function. All of the compounds which inhibit mast cell histamine release also inhibit Ca^{2+} ATPase while morin and rutin are hardly inhibitory in either system.

Racker suggested that the transport ATPases of cell membranes and the associated ion flux pathways are separate structural entities, which when coupled together constitute the ATP dependent ion pumps⁸. In certain tumour cell lines the ion transport functions are apparently uncoupled from the ATPase functions⁸ and to maintain the intracellular ionic milieu, ATP consumption rises. The consequent availability of ADP results in the characteristic enhancement of glycolysis and depressed pH (ref. 22) of some tumour tissues (Warburg effect). When treated with quercetin the efficiency of the transport ATPase is restored and the glycolytic rate declines⁸ although quercetin is not an inhibitor of any of the glycolytic enzymes²³. Quercetin also improved the Ca^{2+} -to-ATP ratio in reconstituted sarcoplasmic reticulum preparations⁸.

Our results suggest that some flavones which affect the efficiency of membrane transport ATPases also prevent Ca^{2+} influx into ligand-stimulated mast cells, raising the possibility that the inhibitor is active at a common locus in both systems. We suggest that Ca^{2+} movements, that is, the efflux due to Ca^{2+} pumping, and influx which follows cross linking of surface receptors, share a common pathway. In the resting cell, the pathway is tightly coupled to the ATPase, but when the cells are treated with cross-linking ligands, the ATPase becomes uncoupled transiently from the flux pathway and there is a net movement of Ca^{2+} down its concentration gradient into the cell. Quercetin could prevent the ligand-induced uncoupling of the ATPase. The inhibitory effect of cyclic AMP on ligand-induced histamine release could also be exerted at the Ca^{2+} ATPase as cyclic AMP is known to modulate the effect of this enzyme²⁴.

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Membrane defect affecting water permeability in human epilepsy

McQUARRIE wrote that a disturbance in water balance perhaps affecting the central nervous system more specifically seems to be closely identified with the aetiology of epilepsy¹. McQuarrie and Teglbjaerg² studied water balance in relation to seizure frequency, establishing the icogenic effect of excessive water ingestion, and the therapeutic value of dehydration. Schneider³ has reported that exacerbation of *petit mal* in children and adults is associated with reduction in urinary volume, and that clinical remission coincidences with an increase in water excretion. Reynolds⁴ has demonstrated disturbances in the whole body distribution of water in epilepsy, with a magnitude proportional to the frequency of attacks, concluding that the changes in body water and sodium are linked in some way to the aetiology of the disease. Wender and Strzyzewski⁵ have shown that water loading in epileptics produces seizures only in cases where there is also a marked increase in the water content of erythrocytes. There is thus abundant evidence linking epilepsy with water metabolism. Furthermore, diuretics, such as acetazolamid, are used in the therapy of epilepsies⁶. But the mechanism of the disturbance of water metabolism at the subcellular and molecular level has not been considered. We report here an investigation of water diffusion through erythrocyte membranes in epileptic children, made using a nuclear magnetic resonance (NMR) technique⁷. We found an abnormally low permeability in membranes from epileptics and suggest that a generalised membrane defect affecting water permeability is responsible for the disturbances of water metabolism in human epilepsy.

The NMR method has been applied to the study of water transfer across nerve cell membranes by Fritz and Swift⁸. The method involves addition of a paramagnetic solution (MnCl_2) to the plasma and measurement of the spin-spin relaxation time (T_2) of the erythrocyte water proton. The spin-spin relaxation time of water inside the isolated erythrocytes is about 140 ms and is much longer than the time required for water to penetrate the membrane (the water exchange time, T_{aw}), which is about 10 ms. If the relaxation time in plasma is made much shorter than the exchange time by adding the paramagnetic ion Mn^{2+} , the observed relaxation time of the erythrocyte (T_{2b}) is dominated by the exchange process through the membrane. T_{2b} is evaluated from a logarithmic plot of the nuclear spin echo as a function of the time between radiofrequency pulses⁹. T_{aw} is calculated as described by Conlon and Outhred⁷. The value of T_{aw} is inversely related to the water permeability of erythrocytes.

Table 1 Clinical features and therapy of epileptic patients

Patient	Age (yrs)	Sex	Classification	Epilepsy Severity	Age of onset (yr)	Drugs and duration of treatment (yr)
1 F.L.	2	F	Idiopathic GM	A	0.1	Phb,Dph (2)
2 M.N.	5	M	Idiopathic GM	A	3	Phb,Dph (2)
3 B.C.	7	F	Idiopathic GM	C	1	Phb,Dph (6)
4 M.V.	9	M	Idiopathic GM	A	7	Phb,Dph (2)
5 P.A.	12	F	Idiopathic GM	A	8	—
6 B.I.	11	M	Idiopathic GM	A	10	Phb,Dph (1)
7 M.V.	10	M	Idiopathic GM	A	7	Phb,Dph (3)
8 M.C.	10	F	Idiopathic GM	A	6	Phb (4)
9 P.N.	12	M	Idiopathic GM + PM	A	9	—
10 P.P.	12	F	Idiopathic PM	A	6	Phb (3)
11 M.C.	14	M	Idiopathic GM	A	4	Phb,Dph (10)
12 G.A.	17	F	Idiopathic GM	B	15	Phb,Dph (2)
13 S.N.	15	F	Focal TL	A	13	Phb (1)
14 L.I.	16	M	Focal TL	A	15	—
15 B.M.	0.8	M	Focal GM	A	0.4	Phb (0.4)
16 B.A.	1	M	Focal GM	A	0.5	Phb (0.5)
17 G.V.	1	F	Focal M	B	0.1	—
18 C.C.	5	M	Focal GM (Gaucher's disease)	A	4	Phb,Dph (1)
19 N.H.	3	M	Focal GM	A	2.7	—
20 N.I.	5	F	Focal GM	C	2	Phb,Dph (3)
21 M.D.	6	M	Focal M	A	4	Phb,Dph (2)
22 L.V.	9	F	Focal M	C	9	Phb,Dph (0.3)
23 B.S.	10	M	Focal GM	A	8	Phb (2)
24 B.L.	10	M	Focal GM	A	9	Phb,Dph (1)

GM, *grand mal*; PM, *petit mal*; M, minor attacks; TL, temporal lobe; Phb, phenobarbital and Dph, diphenylhydantoin. Severity is graded as A, severe (attacks either daily or on most days of the week); C, mild (attacks less than once a month); the remainder were classified as moderate, B.

Table 2 Increases of water exchange time through erythrocyte membranes in epileptic patients

Patient	Blood sample no.	Temperature (°C)	Method	Free Mn ²⁺ concentration in plasma (mM)	ΔT_{ac} %	Mean* ΔT_{ac} (%)
1 F.L.	1	37	CPMG	19.5	21.8	21.7
	2	37	CPMG	19.5	23.0	
	3	37	CPMG	19.5	19.8	
	4	37	CPMG	19.5	22.0	
	5	37	CPMG	19.5	21.9	
2 M.N.	1	20	90° t 180°	9.5	14.5	15.2
	2	20	90° t 180°	9.5	16.0	
3 B.C.	1	20	90° t 180°	9.5	12.5	11.5
	2	20	90° t 180°	9.5	10.5	
4 M.V.	1	20	90° t 180°	9.5	30.4	29.9
	2	37	90° t 180°	19.5	29.5	
5 F.A.	1	37	CPMG	19.5	35.6	36.3
	2	37	CPMG	19.5	37.0	
6 B.I.	1	37	90° t 180°	19.5	20.3	16.3
7 M.V.	1	20	90° t 180°	9.5	32.0	
8 M.C.	1	20	90° t 180°	9.5	17.5	
9 P.N.	1	37	CPMG	19.5	15.5	
	2	37	CPMG	19.5	25.0	
10 P.P.	1	37	CPMG	19.5	32.0	13.3
11 M.C.	1	37	CPMG	19.5	19.5	
12 G.A.	1	37	90° t 180°	19.5	10.5	
13 S.N.	1	20	90° t 180°	9.5	4.6	
14 L.I.	1	20	90° t 180°	9.5	14.4	
15 B.M.	1	20	90° t 180°	9.5	23.4	47.0
16 B.A.	1	37	90° t 180°	19.5	34.6	
17 G.V.	1	20	90° t 180°	9.5	4.6	
18 C.C.	1	20	90° t 180°	9.5	46.0	
	2	20	90° t 180°	9.5	48.0	
19 N.H.	1	20	90° t 180°	9.5	5.7	12.0
20 N.I.	1	37	CPMG	19.5	12.0	
21 M.D.	1	37	CPMG	19.5	7.9	
22 L.V.	1	20	90° t 180°	9.5	18.7	
23 B.S.	1	20	90° t 180°	9.5	21.4	
24 B.L.	1	20	90° t 180°	9.5	15.0	

Mean $\pm$ s.e.m. $19.8 \pm 2.2\%$.

The sample was made by mixing 1 ml of heparinised blood and 0.5 ml of 20 or 40 mM MnCl₂ solution in 0.15 M NaCl. NMR measurements were performed with a Bruker-Physik SXP spectrometer, at 90 MHz. The 90° pulse had a width of 5.5 μ s. The spin-spin relaxation time was measured by the 90° t 180° method and the CPMG method with a pulse sequence spacing of 0.8 ms. Phase detection was used in both cases, and the field was stabilised with the BSN 15 external field stabiliser. The temperature unit of the spectrometer was adjusted to $\pm 0.5^\circ\text{C}$. Measurements were made within several min of preparation of the sample. Blood samples from the epileptic and control children were handled simultaneously to assure comparable conditions. The results are expressed as percentage increase of the water exchange time ($\Delta T_{ac}\%$) in epileptic subjects, the reference being the normal blood. The reference values of T_{ac} for the normal blood were for the 90° t 180° method: 11.1 ms at 20 °C and 9.5 mM Mn²⁺ in plasma, and 8.2 ms at 37 °C and 19.5 mM Mn²⁺ in plasma. For the CPMG method T_{ac} = 8.7 ms at 37 °C and 19.5 mM Mn²⁺ in plasma.

Patients were 24 epileptic children aged 1–17 yr; their main clinical features are summarised in Table 1. An equal number of children aged 2–16 yr formed the control group.

Table 2 shows that in all epileptic children the exchange time of water through the red blood cell membranes was longer than in control subjects. The difference between T_{ae} in epileptic patients and controls was always positive, although measurements were made at different temperatures and concentrations of Mn^{2+} , and two methods were used to measure spin-spin relaxation time—the $90^\circ/180^\circ$ method⁷ and the Carr–Purcell–Meiboom–Gill (CPMG) method (which eliminates errors due to the inhomogeneity of the magnetic field and the molecular diffusion in field gradients⁸). It seems that the water permeability of erythrocyte membrane in epilepsy is less than in the normal situation. There were no significant differences in T_{ae} values between idiopathic and focal epileptics. High values of T_{ae} were noted in patients who suffered from attacks every day and in whom the attacks were poorly controlled by anticonvulsant drugs. But the value of T_{ae} during the attack was not higher than in the interictal period. This indicates that the low water permeability of erythrocytes in epilepsy is not transient but represents a permanent alteration. The abnormal water permeability was not related to the anticonvulsant therapy as it was noted in both untreated and treated patients.

Possible explanations for the observed difference in water proton transfer across the cell membranes in epileptics and normal subjects are an alteration of the erythrocyte permeability affecting water diffusion, and osmotic changes following the addition of the 0.15 M NaCl solution to the blood sample. Any osmotic changes would occur very quickly after mixture of the blood with the physiological saline solution. Consequently, such changes could not influence NMR measurements made several min after the blood was mixed with 0.15 M NaCl. Slow osmotic changes can be excluded because exchange time values remained unchanged for hours, both for normal subjects and epileptics. Alteration of erythrocyte permeability affecting water diffusion is thus the most likely explanation for our finding.

Abnormalities in functions of erythrocyte membranes are considered to indicate a generalised membrane defect^{10,11}. Within certain limits a close qualitative and quantitative analogy is possible between the stability of the membrane of the erythrocyte and that of the neurone^{12,13}. Fritz and Swift⁴ have shown (by the NMR method we used) that for the frog nerve the exchange rate of the water protons between the intra- and extracellular environments is lower for the depolarised nerve than for the polarised nerve. That is, the water permeability of the membrane is smaller in the depolarised state than in the resting state.

The polarisation of the membrane is due to the differential distribution of Na and K across the excitable neuronal membranes, maintained by the Na–K-activated ATPase¹⁴. It has been suggested¹⁵ that the final common expression of epileptogenicity is an impairment of operation of the Na and K transport systems in epileptogenic neurones, which creates the characteristic instability of the excitable membrane. Harris¹⁶ has suggested that changes in distribution or transport of Na^+ and K^+ may not be due only to effects on the energy state or on ATPase activity, but may be a reflection of a generalised defect of membrane permeability.

We suggest that decreased water permeability in erythrocytes of epileptics may reflect a membrane defect in all tissues and may be an expression of the individual predisposition to epilepsy; it might be of particular importance in the nervous system. Further studies on erythrocyte membranes in epilepsy may give clues to the understanding of the membrane defect in molecular terms. The erythrocyte may be a useful model for testing *in vivo* the possible effectiveness of treatment with anticonvulsant drugs in individual patients, or for testing new anticonvulsant drugs.

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New human embryonic antigen and its presence in some adenocarcinomas

IMMUNODIFFUSION methods have identified several embryonic antigens in tumour extracts or in sera of patients with primary cancer of the liver^{1–3}, colonic adenocarcinoma^{4–6}, testicular and ovarian teratoblastomas^{7,8}, carcinoma of the pancreas⁹, chorionepithelioma of the uterus¹⁰ and testicular chorioncarcinoma¹¹, nephroblastoma¹² and adenocarcinoma of the ovary^{13,14}. We report here the use of monospecific antisera to identify a new embryonic antigen in human foetal sera and in some adenocarcinoma extracts.

Antisera against extracts of human ovarian adenocarcinomas were prepared in rabbits¹⁵. After absorption by human pooled plasma (20–40 mg ml⁻¹) and pooled extracts

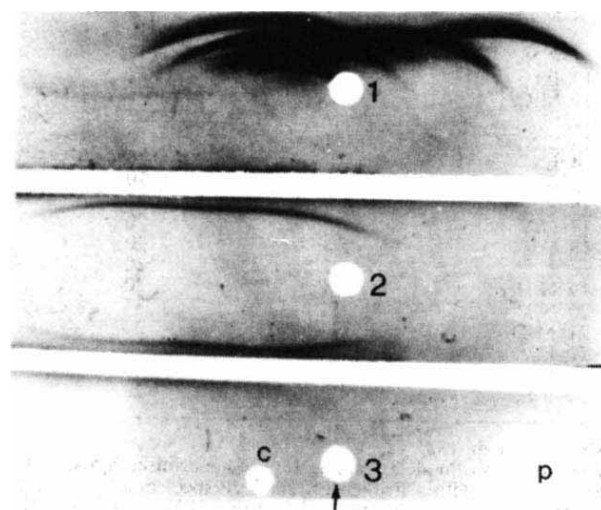


Fig. 1 Gel immunoelectrophoresis of human foetal and adult sera. Precipitation lines were developed with antiserum against human serum proteins¹ and with adsorbed antiserum against ovarian adenocarcinoma extract². Sera of: 1, 3, human pregnancy sera; 2, foetus of 20 weeks. Zone of migration: c, colour T-1824; p, pyronine.

Table 1 Immunodiffusion of absorbed antisera against various sera and tissue homogenates

Sample	No. of samples examined	Results of gel diffusion analysis		Titre
		No. positive	No. negative	
Human serum of				
Foetuses of 20–30 wk	20	20	0	1:8–1:32
Foetuses of 30–40 wk	11	11	0	1:2–1:8
Newborns of 30–40 wk	20	20	0	1:1–1:2
Adult donors (male and female)	50	0	50	—
Tissue homogenates of				
Ovarian adenocarcinoma	26	13	13	1:1
Colonic adenocarcinoma	17	7	10	1:1
Carcinoma of stomach	16	3	13	1:1
Uterine carcinomas	9	0	9	—
Lung carcinoma	5	0	5	—
Miscellaneous	10	0	10	—
Normal adult, various organs*	36	0	36	—

*Included ovary, testis, liver, spleen, pancreas, kidney, gastrointestinal tract, brain and muscle tissue.

of normal tissues ($40\text{--}60\text{ mg ml}^{-1}$) the antisera were used for immunoelectrophoresis¹⁵ and immunodiffusion in agar¹⁶. The newly discovered antigen migrated with α -globulin and prealbumin in immunoelectrophoresis (Fig. 1). Similar electrophoretic fractions are present in all human embryonic sera. Immunodiffusion analysis revealed analogous antigen in sera of newborns as well as in some individual extracts of ovarian and colonic adenocarcinomas (Fig. 2) but not in adult sera or in other normal and malignant tissues. These data are summarised in Table 1.

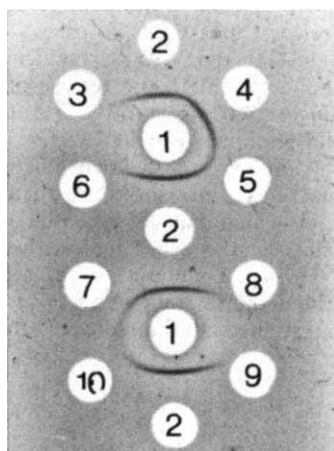


Fig. 2 Gel double diffusion with standard test system: 1, monospecific antiserum; 2, standard antigen. Human serum of 3, adult donor; 4, foetus 20 weeks; 7, newborn. Homogenates of 5,6, ovarian adenocarcinomas; 8, hypernephroid tumours; 9,10, colon carcinomas.

Comparative immunodiffusion showed that the reported embryonic antigen was not identical to α -foetoprotein¹⁷, carcinoembryonic antigen⁴, bovine fetuin¹⁸, or pregnancy-specific β -1-globulin¹⁹.

The results suggest that the newly identified antigen is a serum protein secreted during embryonic development, and can be synthesised in adults with adenocarcinomas. The physiological role and cellular localisation of this antigen are not known.

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Reconstitution in phospholipid vesicles of a glucose transport system from pig small intestine

ABSORPTION of monosaccharides from the small intestine in mammals occurs by several different processes. The major barrier to absorption is the brush border membrane of the epithelial cells across which glucose is transported against its concentration gradient in a sodium-coupled transport system. We describe here some properties of monosaccharide transport from phospholipid vesicles into which brush border proteins obtained from neonatal pig small intestine were incorporated. The system transported glucose, but not fructose more rapidly in the presence of a sodium gradient; it transported D-glucose more rapidly than L-glucose and efflux was reduced by phlorizin.

Small intestines were removed from unfed newborn pigs from the Babraham herd. All biochemical procedures were performed in a cold room at 5 °C. Preparations of small intestinal brush border membranes were made by a modification of the method of Porteous¹; purity was checked by phase-contrast microscopy. There was no reaction to the diphenylamine reagent test for DNA in brush-border samples. Table 1 shows the results of enzymatic assays on subcellular fractions. Although there was little mitochondrial contamination there was evidence of acid phosphatase release from lysosomes. The preparation contained some baso-lateral membranes. Brush border proteins were either immediately incorporated into phospholipid vesicles, or stored deep frozen for up to 2 months before use, with small loss in glucose-transporting activity.

Phospholipid vesicles were prepared from purified soya bean phospholipids using cholic acid, the modification by

Table 1 Enzymatic activities in small intestines of newborn pigs

Enzyme	Crude homogenate	Mitochondrial fraction	Brush-border fraction
Monoamine oxidase ^a (nmol tyramine oxidised h ⁻¹ mg protein ⁻¹)	200.9	318.6	189.2
Succinic dehydrogenase ^a (μmol 2-6 dichlorophenolindophenol oxidised h ⁻¹ mg protein ⁻¹)	7.04	31.4	10.5
Acid phosphatase ^a (μmol <i>p</i> -nitrophenylphosphate metabolised h ⁻¹ mg protein ⁻¹)	212.8	976.2	1872.2
Sucrase ^b (μmol metabolised h ⁻¹ mg protein ⁻¹)	—	—	< 50

Warren *et al.*⁶ of Racker's⁷ method. Vesicles containing brush border protein were prepared in sodium-sucrose buffer (pH 8.0) containing 250 mM sucrose, 1 M NaCl, 50 mM NaH₂PO₄ and 2 mM glucose, or in other experiments, appropriate monosaccharide mixtures or phlorizin. Cholic acid was dialysed away overnight against the same buffer at pH 7.2. The sugar was labelled by allowing exchange with an appropriately labelled radioactive sugar in the medium for at least 30 min before each experiment. Initial experiments established the optimum ratio of phospholipid:cholic acid:brush border protein in the mixture before formation of the vesicles, as 60:30:1, a proportion similar to that used by Warren *et al.*⁶. Transport of sugars from the vesicles was measured by holding them within the cellulose hollow fibres of a Bio-Rad Bio-Fiber Mini-beaker. These fibres allow compounds of molecular weight < 5000 to diffuse freely into the fluid which was pumped through the beaker at a constant rate of 4.5 ml min⁻¹. Aliquots of perfusate were collected with a fraction collector and radioactivity measured by liquid scintillation spectrometry. The fluid used to perfuse the fibres was either of the same composition as the fluid against which the vesicles were dialysed (though containing neither glucose nor fructose) or a potassium-sucrose buffer containing identical amounts of equivalent potassium salts. Normally each perfusion involved an initial period during which sodium-sucrose buffer was perfused through the

beaker and collected, and a second period of perfusion with potassium-sucrose buffer. Radioactive glucose or fructose released from the hollow fibres during the first period was from two sources—extravesicular fluid, and from inside the vesicles flowing down a concentration gradient. In the second period, amounts of monosaccharide from these first two sources were small, the majority being released from the vesicles when there is a sodium gradient from within the vesicles to the bathing fluid.

Figure 1 compares the release of glucose in vesicles containing brush border protein with those without protein. In Fig. 2 the outflow of glucose is seen in the absence of a sodium gradient together with the relative outflows of D- and L-glucose in the presence of a sodium gradient, D-glucose being transported more readily than its isomer. Total glucose release measured in tubes 40–60 inclusive was 3.11 μg D-glucose and 1.95 μg L-glucose (ratio D:L, 1.59). In a further experiment the release of D-glucose was 5.02 μg and L-glucose 4.07 μg (ratio 1.23). In both of these experiments phospholipid vesicles were loaded with a mixture of equal amounts of the two glucose isomers and the simultaneous release measured. A further experiment was performed in which half of a batch of vesicles was loaded with D-glucose, the other half with L-glucose; here 10.66 μg and 6.67 μg L-glucose were released by the application of the sodium gradient (ratio 1.60).

Murer *et al.*⁸ studied the uptake of the two glucose

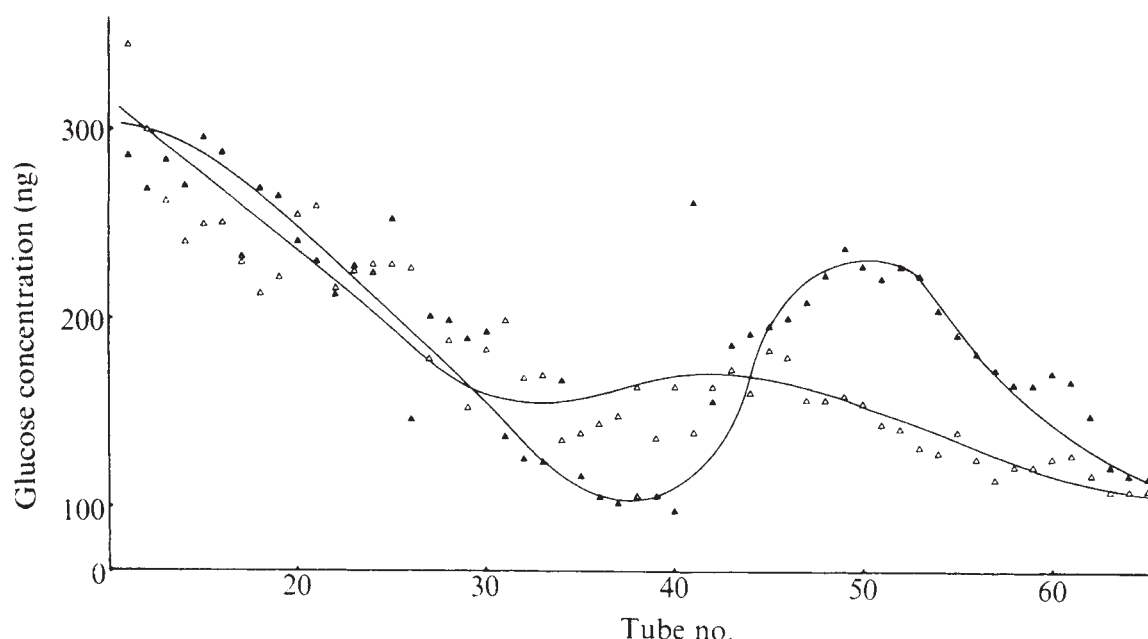
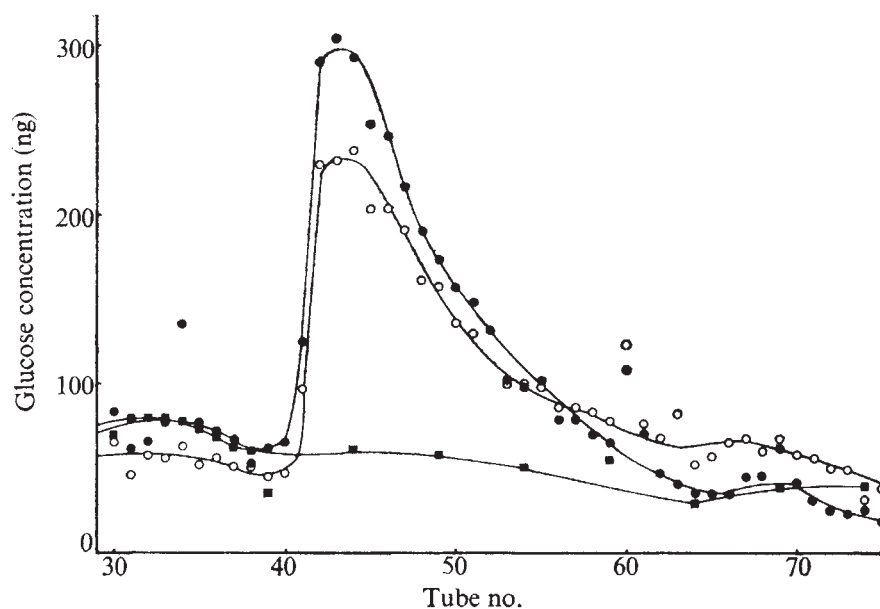


Fig. 1 Glucose release from phospholipid vesicles containing brush border protein (▲) and containing no protein (○). The sodium gradient was introduced at tube 30 by perfusion with potassium-sucrose buffer. The delay in appearance of glucose from the vesicles containing protein is due to the deadspace in the system. Total glucose released from the protein-containing vesicles between tubes 40 and 60 was 4.03 μg, from those without protein 3.02 μg.

Fig. 2 Glucose release from phospholipid vesicles; *a*, in the absence of a sodium gradient (■); *b*, after loading with a mixture of D- and L-glucose, D-glucose being labelled with ^3H (●) and L-glucose with ^{14}C (○). The sodium gradient was introduced at tube 30.



isomers into isolated brush border vesicles using a filtration method; although after 120 s D-glucose uptake was 3.3 times greater than L-glucose, the ratio after 25 min was 1.4, similar to that seen in the present experiments.

Phlorizin (which inhibits transport of glucose both in small intestine and the renal proximal tubule) was incorporated into phospholipid vesicles at 5×10^{-4} M together with 2 mM glucose, and efflux of glucose was compared with controls containing no phlorizin (Fig. 3.) The initial loss of glucose from the vesicles down its chemical gradient was higher in the control than in the presence of phlorizin. After application of the sodium gradient, 0.84 μg of glucose was lost from the controls and 1.26 μg from the vesicles with phlorizin, the greater loss from the latter experiment probably being due to the larger amount of glucose which was still present in those vesicles. Total loss of glucose from control vesicles during the complete experiment was 2.7 μg , and in the presence of phlorizin, 1.9 μg . Fructose is not absorbed from the intestine by a sodium dependent mechanism, so the efflux of this sugar was compared with

that of glucose. Vesicles were loaded with a mixture of ^3H -glucose 1.5 mM and ^{14}C -fructose 1.5 mM. Three perfusions were performed and the amounts of glucose released during the peak after the addition of the sodium gradient were 5.6, 5.1 and 5.14 μg , and of fructose, 2.11, 0.89 and 2.79 μg respectively. Furthermore, there was no significant rise in fructose efflux when the sodium gradient was introduced (Fig. 4).

Fig. 3 Release of D-glucose from phospholipid vesicles; *a*, control (●) and *b*, in presence of 5×10^{-4} M phlorizin (○). Vesicles were perfused with sodium sucrose buffer from samples 1–10. At the arrow it was changed to potassium-sucrose buffer. As in Figs 1 and 2 peaks show glucose release in response to the production of a Na^+ gradient.

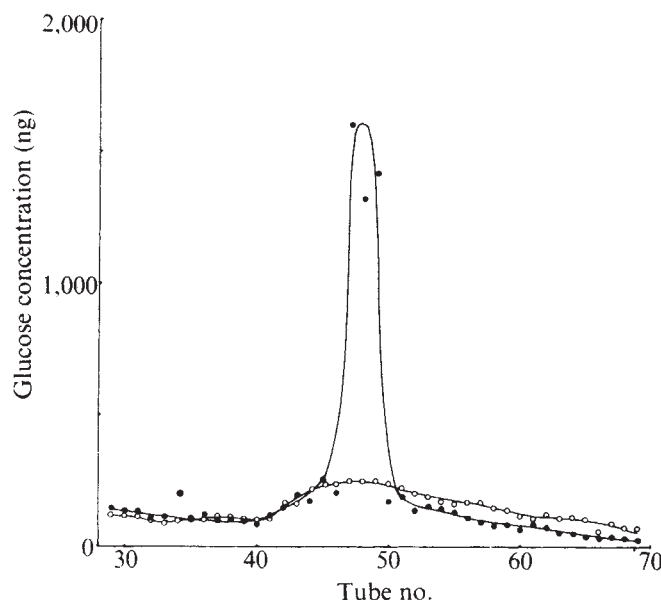
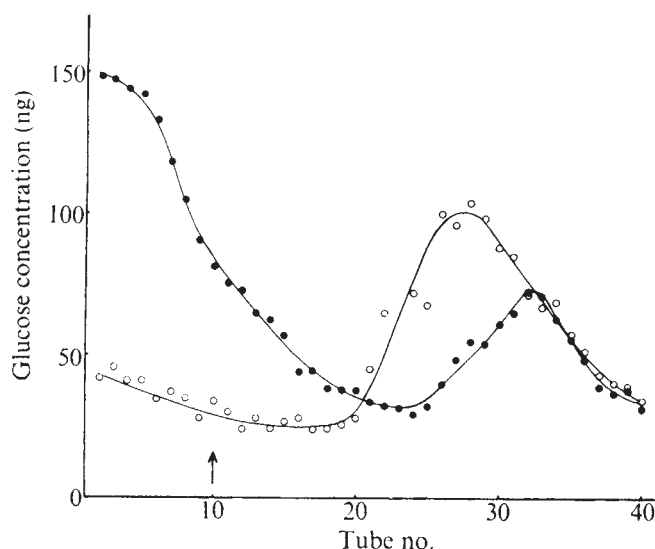


Fig. 4 Release of D-glucose (●) and fructose (○) from phospholipid vesicles in the presence of a sodium gradient introduced at tube 30.

The results of experiments using L-glucose and fructose might be explained if exchange of the labelled compounds with unlabelled sugar in the vesicles was not complete at 30 min. This is unlikely for three reasons. First, there was no change in the relative efflux of the two compounds when vesicles had been in contact with the isotope for varying times in excess of the initial 30 min. Second, the initial period, which consists at least in part of sugar from inside the vesicles, was very similar in D-glucose, L-glucose and fructose-loaded vesicles. Third, after the large sodium-

dependent efflux of D-glucose the depleted vesicles released less than the vesicles containing a sugar, for example, L-glucose more resistant to release by a sodium gradient, so that in Fig. 1 the efflux of D-glucose crosses that of L-glucose after the peak efflux has occurred, and this was a consistent finding.

Our results show that a glucose transporting system can be reconstituted from phospholipid vesicles and protein from brush border membranes from small intestinal cell brush borders from pigs. This transport resembles the characteristics of transport of glucose in intact tissue in that it is increased in the presence of a sodium gradient, whereas the transport of a closely related sugar, fructose, is not. Furthermore, the reconstituted system distinguishes D-glucose from the L-isomer, and the total efflux of glucose is also reduced by phlorizin.

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Immunofluorescence shows localisation of prolactin to human amnion

AMNIOTIC fluid in man and rhesus monkey contains very high concentrations of prolactin¹⁻³, but its origins are unknown. It does not seem to come from the maternal circulation because isotopically labelled prolactin injected into the pregnant rhesus monkey does not appear in amniotic fluid². One source of prolactin is suggested by the passage of foetal pituitary prolactin through the immature kidney into the urine and then into amniotic fluid^{4,5}. But amniotic fluid and foetal prolactin differ in molecular size, suggesting different origins⁴. Incubated human amnion and chorion, but not placenta, release prolactin into the medium, indicating a second source of amniotic fluid prolactin². Using indirect immunofluorescence, we report localisation of prolactin to the human amnion.

Placentae and amniotic membranes were collected from normal pregnancies of 12-40 weeks gestation, which had been terminated for psychiatric reasons in the second trimester by hysterotomy or had been delivered vaginally either prematurely or at term in the third trimester. The tissue was immediately cooled in ice and rinsed clean of blood and amniotic fluid with phosphate-buffered saline (pH 7.4). Within 90 min of delivery, tissue blocks (1 cm³) of cotyledon, umbilical cord, placental and reflected parts of the amnion and the chorion laeve were snap frozen in isopentane liquid nitrogen at -70 °C. Specimens were also fixed in 10% phosphate-buffered formalin for conventional histology.

Frozen sections, 6 µm thick, were cut on a Lipshaw cryostat and examined by indirect immunofluorescence⁶ using rabbit antisera to human prolactin obtained from the National Institutes of Health, Bethesda, Maryland. The

reaction was traced with a fluorescein isothiocyanate-labelled goat anti-rabbit γ-globulin. The unabsorbed conjugate had a fluorescein to protein molar ratio of 4.8 : 1 and was absorbed with rat liver, kidney, gastrointestinal tract, sheep liver and human placenta so that it gave no nonspecific staining. The absorbed conjugate was used at a protein concentration of 8 mg ml⁻¹. After staining, the sections were examined by dark ground ultraviolet fluorescent microscopy using a condenser fitted with a colourless barrier filter and a toric lens beneath.

Immunological specificity of the reaction was assessed by reacting parallel control sections with conjugate alone, normal rabbit serum or rabbit anti-human prolactin neutralised by absorption with human prolactin, growth hormone or chorionic somatomammotrophin (placental lactogen). For neutralisation studies, 50 µl of rabbit anti-human prolactin was incubated overnight with 100 µl of human prolactin at a concentration of 6 µg ml⁻¹. The other hormones were used at similar concentrations. The rabbit anti-human prolactin serum was examined in a radio-immunoassay, and did not bind human growth hormone, insulin, chorionic somatomammotrophin or gonadotrophin, pituitary thyrotrophin and gonadotrophins or prostaglandins F_{2α} or E₁.

Multiple sections from seven pregnancies stained with rabbit anti-human prolactin showed specific immunofluorescence localised to the cytoplasm of the amnionic epithelium. Figure 1 demonstrates the uniform staining reaction throughout the cytoplasm of all epithelial cells. The intensity



Fig. 1 Cytoplasmic immunofluorescence of a fold of human amnion epithelium from a 28-week pregnancy stained by rabbit anti-human prolactin. Small blood vessels are also stained (× 572).

of immunofluorescence varied with the amnion of different patients and in general was more intense in the second trimester, but there seemed to be little variation in staining reaction between the placental and the reflected amnion from the same patient. In some sections, occasional blood vessels deep to the amnionic epithelium showed positive staining but this was never as intense as the staining of the overlying epithelium. No immunofluorescence was observed in the chorion laeve, umbilical cord or placental chorionic villi. Control section of amnion failed to stain when tested with conjugate alone or with normal rabbit serum. Anti-serum absorbed with growth hormone or chorionic somatomammotrophin stained the amnionic epithelium but anti-serum absorbed with human prolactin did not stain.

We have demonstrated that prolactin is localised in the cytoplasm of human amnionic epithelium. It is possible that prolactin synthesis occurs in these cells, thus contributing to the relatively high concentrations of prolactin in amniotic fluid. The amnion epithelial cells are metabolically active⁷ and contain rough endoplasmic reticulum capable of protein synthesis^{8,9}. Although prolactin receptors have not been reported in human placental membranes or myometrium, this hormone influences water and electrolyte change in many species including man and may affect the volume and composition of human amniotic fluid¹⁰⁻¹²; in addition, it inhibits myometrial contraction¹³. Local production or concentration of prolactin by the epithelial cells of the amnion would facilitate these functions.

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Palatal cytosol cortisol-binding protein associated with cleft palate susceptibility and *H-2* genotype

GENETICALLY different inbred strains of mice have different susceptibilities to the production of cleft palate by a standard dose regimen of corticoids at the critical period during palatal organogenesis¹. Because in target tissues corticoids form a specific cytoplasmic cortisol-receptor complex which interacts with the genome, resulting in activation or depression of transcription^{2,3}, we have postulated that susceptibility to corticoid-induced cleft palate is mediated by genetic differences in a cytosolic corticoid receptor protein(s) in palatal anlagen⁴. Other studies have suggested that susceptibility to cortisone-induced cleft palate is regulated by genes closely linked to the *H-2* locus⁵. We report here that the total level of cytosolic protein binders of ³H-cortisol

in maternal and foetal palates on day 11 of gestation correlates with susceptibility to cleft palate and with the *H-2* genotype. After separation of macromolecular bound hormone from free hormone on columns of Sephadex G-25 and further separation by microelectrofocusing⁶, only one maternal and foetal palatal cytosol binder for ³H-cortisol (*pI* 6.9-7.0) was found to correlate with susceptibility to cleft palate and the *H-2* genotype. Thus, a product of a gene in or near the *H-2* locus seems to be the glucocorticoid receptor, the level of which must be increased for a congenital malformation to occur.

The percentages of offspring in the different strains with cleft palate after maternal administration of corticoids were A/J, 100, DBA/1J, 92; C3H/HEJ, 68; C57BL/6J, 19 and CBA/J, 12, respectively¹. This production of cleft palate by corticoids has been associated with the *H-2* genotype by the observation that the incidence of cortisone-induced cleft palate in C57BL/10 ScSn (B10) is 21%, and that of A/J is 100%; whereas that of B10.A, which has the genetic background of B10, but the *H-2*^a allele of A/J, is 81% (ref. 5). Foetuses of the sensitive A/J strain retain more ¹⁴C-cortisone during the critical period than do those of the resistant strains, CBA/J (ref. 7) or C57BL/6J (ref. 8). Furthermore, synthetic corticoids are recovered in the unmetabolised state from foetal A/J tissues¹⁰ and inhibit foetal RNA and protein synthesis at the critical period of cleft palate production¹¹. A/J foetuses are more sensitive to an inhibition of RNA synthesis than those of the C3H/HEJ strain¹² and the effect is not due to a difference in metabolism of the steroid by A/J and C3H/HEJ strains¹⁰.

A typical microelectrofocused pattern of ³H-cortisol-binding proteins in the foetal jaws of each of the five inbred strains is shown in Fig. 1. Only one ³H-cortisol-binding protein increased in amount in the different strains in proportion to the reported incidence of cleft palate produced by the standard cortisone regimen (indicated by arrows) (Fig. 1).

Most of the ³H tightly bound to protein in the cytosol and nuclei of the foetal jaws was unmetabolised ³H-cortisol (Fig. 2).

A linear regression analysis of each peak microelectrofocused showed a highly significant correlation between the amount of ³H-cortisol-binding protein and the reported incidence of cleft palate in the seven strains: there was only one peak (*pI* 6.9-7.0) in both maternal palates ($r = 0.485$, $n = 27$, $P < 0.015$) and foetal jaws ($r = 0.585$, $n = 19$, $P < 0.016$) (Fig. 3).

A linear regression analysis also demonstrated that the total ³H-cortisol-binding proteins in maternal ($r = 0.412$, $n = 30$, $P < 0.025$) and foetal ($r = 0.494$, $n = 18$, $P < 0.042$) palates of each of the seven strains was significantly correlated with the reported incidence of corticoid-induced cleft palate in that strain (data not shown). Moreover, when a pool of five adult male palates each of the B10 and B10.A strains taken 1 h after injection of 10 μ Ci ³H-cortisol was analysed for this ³H-cortisol-binding protein, the values plotted on the same linear regression curve as the maternal palates of the seven strains.

Our results show that the levels of foetal and maternal palatal cytosolic ³H-cortisol-binding proteins (total and *pI* 6.9-7.0) are correlated with susceptibility to corticoid-induced cleft palate and are associated with alleles at the *H-2* locus. The *H-2* locus is not the sole determinant of ³H-cortisol receptor levels, for C3H/HEJ (*H-2*^b) and CBA/J (*H-2*^k) have the same *H-2* genotype but different cleft palate susceptibilities and receptor levels. Our data suggest, however, that the ³H-cortisol-binding protein with *pI* 6.9-7.0 is a candidate for the cortisol receptor in the mouse palate and that susceptibility to cortisone-induced cleft palate is mediated by this candidate for the cortisol receptor. A similar mechanism has been invoked to explain why medroxyprogesterone acetate produces cleft palate in

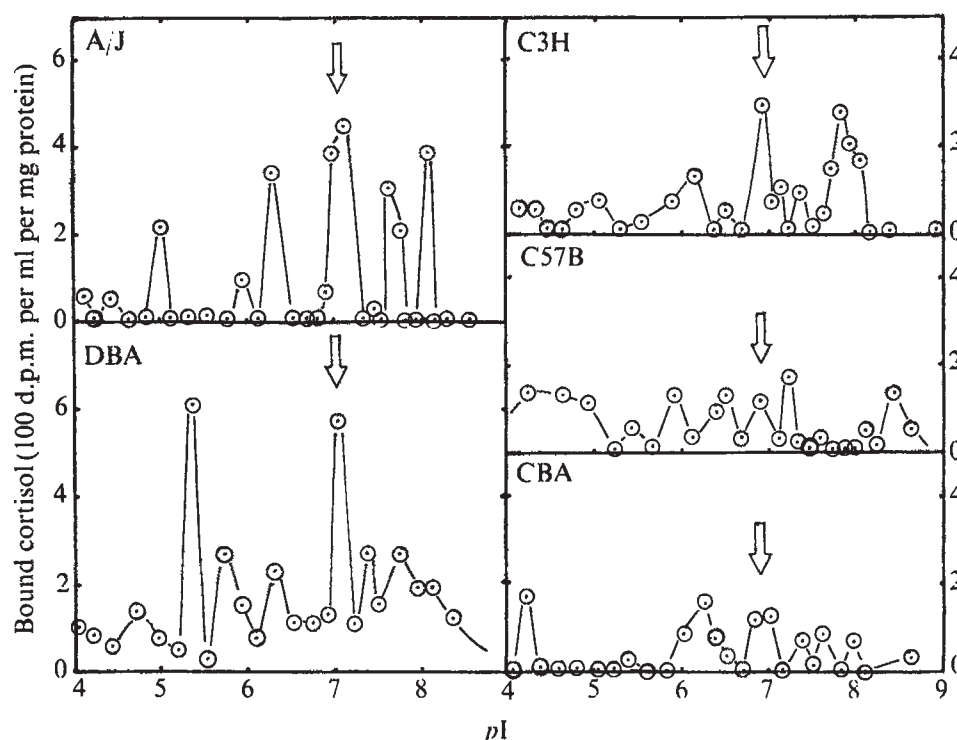


Fig. 1 Typical microelectrofocusing pattern of 1,2,6,7- ^3H -cortisol-binding proteins in cytosol of foetal jaws after gel filtration through Sephadex G-25. Pregnant mice were killed by decapitation at 11 d of gestation (day of vaginal plug = day 0) 1 h after subcutaneous injection of 5 μCi of 1,2,6,7- ^3H -cortisol (90 Ci mmol^{-1}). Maternal palates and the jaw regions (horizontal cut of the foetal head just below the level of the eyes including upper and lower jaws) of foetuses obtained by caesarian section were dissected, pooled (two or three maternal palates per pool and jaws of all foetuses in one or two litters per pool) and homogenised in a fivefold volume of 0.3 M sucrose-1 mM EDTA-20 mM Tris-50 mM phosphate buffer, pH 7.4. Cytosol was prepared, filtered through Sephadex G-25 and microelectrofocused as described before⁶ with the exception that the columns were changed to 25-ml pipette and the Triton X-100 was eliminated. Each 0.5-ml sample was fractionated after electrofocusing at 200 V for 40 h. Radioactive samples

were mixed with Formula 950A (New England Nuclear) and counted in a Packard Tricarb model 3375 scintillation spectrometer with a counting efficiency of 30-40% for ^3H . Quenching was determined by an automatic external Standardisation ratio method. Protein was determined by the method of Lowry *et al.*¹⁷. The arrows indicate the pI 6.9-7.0 binding protein. The amount of this protein is proportional to the reported incidence of cleft palate produced by four daily doses of 2.5 mg cortisone from days 11 to 14 of gestation in the different strains (Fig. 3). Each determination represents a pool of all the foetal jaws in one or two litters.

rabbits, but not in rats, since medroxyprogesterone acetate effectively competed with the binding ^3H -dexamethasone to the rabbit, but not to the rat liver glucocorticoid receptor⁹. We have found ^3H -cortisol largely in the unmetabolised state in the nuclei of the foetal jaws 1 h after maternal injection. This suggests nuclear uptake of a cortisol receptor, which presumably then results in repression or derepression of protein synthesis.

Detailed purification efforts reported so far by two groups

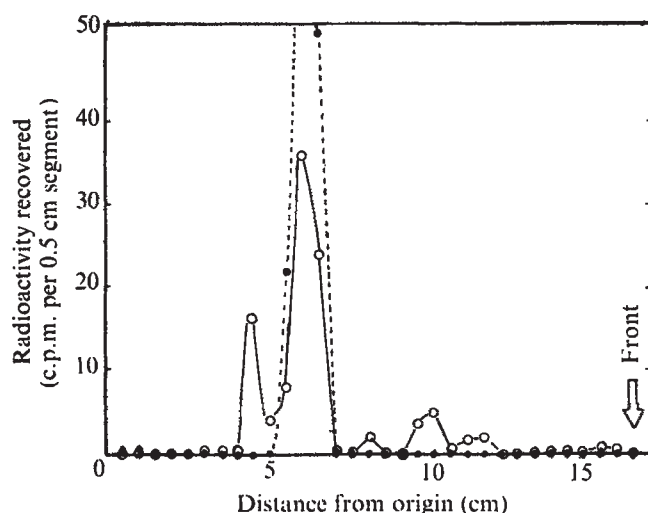
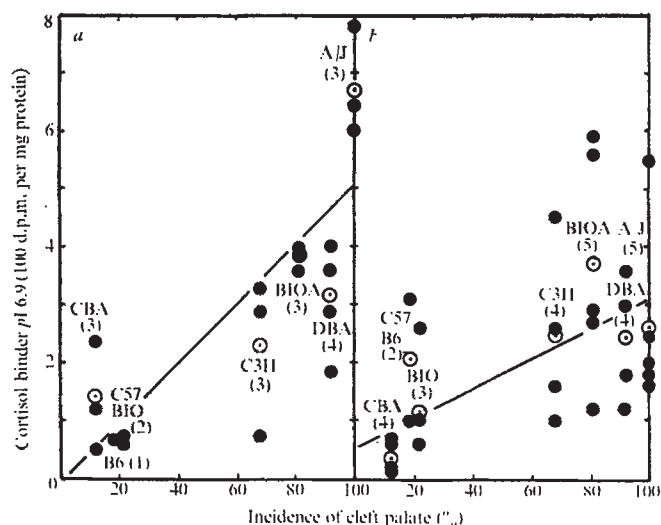


Fig. 2 From 75 to 80% of the ^3H in a 50% methylene chloride-chloroform extract of the cytosol and nuclei of the foetal jaws of the sensitive strains cochromatographed with an equivalent amount of ^{14}C -cortisol in a thin-layer chromatogram developed in cyclohexane-ethyl acetate-ethanol (45:45:10, v/v/v). The remainder of the ^3H was not identified accurately, but had the same mobility in this system as a non-radioactive cortisol metabolite, 3 β , 11 β -17 α -, 21-tetrahydroxy-5 α -pregnane. Circle and dot, ^3H in extract; $\bullet$, authentic ^{14}C -cortisol.

have shown that the major rat^{13,14}, guinea pig¹⁵ and human¹⁶ liver glucocorticosteroid receptor capable of nuclear transfer *in vivo* and *in vitro* has an isoelectric point of either 7.1 and 6.1 after thermal activation¹⁰, or 6.7 (refs 11-13). Other binding fractions found by us and the other groups do not seem to be receptors but transcortin (corticoid-binding globulin), ligandin (possibly an enzyme) or receptor fragments. The similarity of the pI of the ^3H -cortisol-binding protein associated with susceptibility to cleft palate with those of the liver glucocorticoid receptor found by the two groups makes the protein a candidate for a palatal glucocorticoid receptor mediating cleft palate.

Fig. 3 Linear regression analysis of pI 6.9-7.0 receptor level of foetal jaws (a) and maternal palates (b) for each strain compared with reported mean incidence of cleft palate in each strain. The numbers in parentheses indicate the number of pools of adult palates or foetal jaws. Circle and dot, mean value of all pools for each strain; $\bullet$, value of each pool.



The association between *H*-2 genotype and level of cortisol receptor in the cytosol suggests that the protein with *p*_I 6.9–7.0 is either coded by a gene closely linked to the *H*-2 region or coded elsewhere in the genome, but is strongly affected by a gene in or near the *H*-2 region. Thus, the diversity of effects known to be associated with *H*-2 could be explained by a series of closely linked genes adapted to serve either immunological or hormonal functions.

After submission of our paper evidence in agreement with our findings was reported, to the effect that foetal A/J facial mesenchyme cells contain about twice the amount of saturable dexamethasone receptors than C57BL/6J mesenchyme cells obtained either directly or in primary culture¹⁵.

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Neuroanatomical focus for morphine and enkephalin-induced hypermotility

OPIATE receptors, through which all opiate alkaloids and peptides produce their pharmacological effects, are distributed heterogeneously in the mammalian brain^{1–3}. The highest receptor densities seem to be associated with a number of limbic midbrain and forebrain structures that have been implicated in regulating motivation and emotionality. Although the limbic midbrain region (periventricular-periaqueductal grey matter) is a critical focus for the analgesic^{4–7} and depressant (A.P. and C.S., in preparation) effects of morphine and enkephalin⁸, nothing is known

about the functional significance of opiate receptors in the forebrain. Significantly, the nucleus accumbens (NA) in the limbic forebrain contains a relatively high concentration of opiate receptors² (C. B. Pert, personal communication). As this structure has a role in motility^{9,10}, an attempt has been made to ascertain whether it is an important focus for the excitatory effects^{11,12} of opiates on behaviour. We report here that direct injections of morphine, D-Ala¹-enkephalin-amide (an enzyme-resistant enkephalin)^{7,12} and apomorphine (a dopamine agonist) into the nucleus accumbens increased spontaneous motor activity in rats. The excitatory effects of morphine were antagonised by naloxone (an opiate antagonist) and not by haloperidol (a dopaminergic antagonist). Conversely, apomorphine was antagonised by haloperidol and not by naloxone.

Thirty-five male Sprague–Dawley rats, anaesthetised with sodium pentobarbital, were implanted stereotactically with bilateral intracerebral cannulae guides constructed from 23-gauge hypodermic tubing. The guide cannulae tips were aimed for a region 2 mm dorsal to the NA (AP 9.4, LAT 1.5, DV 2.0) (ref. 15). Drugs were injected into the NA through 30-gauge injectors which protruded 2 mm past the guide tips. Drug and control solutions were administered in a volume of 1 μ l and at a rate of 1 μ l per 30 s. One group of 10 animals was injected bilaterally in the NA with 5 μ g morphine sulphate (that is, a 10 μ g total dose), 10 μ g apomorphine and 1 μ l of water (the drug vehicle). All animals received both drugs and the vehicle in a counterbalanced order separated by 7 d. Another group of animals (*n*=9) was injected in the NA with both 10 μ g D-Ala²-enkephalin and 1 μ l of water in a counterbalanced order, as above. Immediately after injections the animals were placed in Plexiglas chambers (23×23×15 cm) which were mounted in Motron motility meters (model 40Fc). Horizontal and vertical activity was monitored in 30-min segments for 6 h. Animals in these studies were always injected at 1000 h and removed from the apparatus at 1600 h. Six additional rats received injections of morphine and the vehicle into a region 2 mm dorsolateral to the NA. These rats were tested 30, 180 and 360 min after injection for 30 min.

Other groups of rats were injected with either 10 μ g of apomorphine or 5 μ g of morphine in the NA. Rats injected with apomorphine were pretreated with either naloxone (10 mg per kg body weight), haloperidol (0.25 mg per kg body weight) or normal saline administered intraperitoneally (i.p.) 15 min before being injected intracerebrally (i.c.). Rats injected with morphine i.c. were injected i.p. with either haloperidol, naloxone or normal saline 165 min later. Rats injected i.c. with morphine were placed in the apparatus 180 min after the i.c. injection. Rats injected with apomorphine were tested immediately after injection. These regimens were adopted to assure that both i.c. drug effects were at asymptotic levels during testing. All animals were tested for 30 min. Two groups of rats were also tested 15 min after i.p. injections of either naloxone and saline or apomorphine and saline. Rats in the above studies were tested between 0900 and 1500 h.

Apomorphine produced an immediate increase in both horizontal and vertical spontaneous activity after NA injections. This hyperactivity dissipated rapidly over 1 h and was no longer detectable 90 min after injection. Morphine, however, induced a more gradual increase in activity which reached asymptotic levels in approximately 3 h and was still present 5.5–6 h after injection. The excitatory effect of D-Ala²-Met-enkephalin also had a gradual onset but was not as persistent as morphine (Fig. 1).

Naloxone pretreatment antagonised the excitatory effects of morphine, whereas haloperidol was ineffective. Apomorphine, on the other hand, was antagonised by haloperidol and not by naloxone. Both naloxone and haloperidol decreased motor activity at the doses tested. Only the

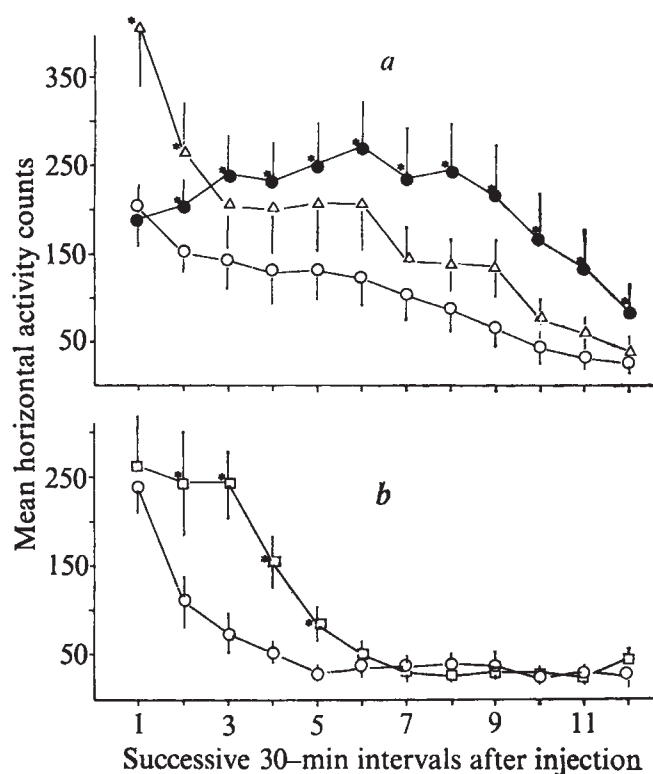


Fig. 1 Time course of horizontal hypermotility after bilateral injections of vehicle alone (○), morphine 5 µg (●), apomorphine 10 µg (▲) or D-Ala²-Met-enkephalin 10 µg (□) into the NA. Repeated measures analyses of variance revealed a significant drug effect for both groups of rats (10 rats in *a*; 9 in *b*). Vertical lines indicate s.e.m. Similar results were obtained for vertical activity. * $P < 0.05$ for comparisons between effects produced by the drugs and the vehicle at each time point.

naloxone-induced hypomotility, however, differed significantly from the saline control level (Fig. 2). This finding seems to suggest the existence in brain of a tonically active enkephalinergic system with a role in modulating some aspects of motor activity.

Standard histological verification of injection sites at the termination of each study revealed that all injections had been made directly into the NA or into regions immediately bordering this structure. Injection sites in the anatomical control group, which had been implanted with cannulae aimed for structures outside the NA, were located in an area 1.5–2.5 mm dorsalateral, and in some cases anterior to, the NA. No hyperactivity was evident in this group after i.c. injections of morphine at any of the time points tested.

Both opiates^{11,12} and various catecholaminergic agonists^{15–17} have been reported to increase spontaneous motility in rodents after systemic injections. The NA, a terminal region for the mesolimbic dopamine pathways originating from area A10, has been implicated as a principal focus for the excitatory actions of amphetamine⁸ and apomorphine^{10,18}. These compounds affect activity either by increasing the availability of dopamine at the synapse, or by a direct action on dopamine receptors, as DA receptor blockers antagonise their excitatory actions. Opiates may also increase motility by activating the catecholamine systems^{19–21}. Our findings, however, indicate that, at least in the rat forebrain, opiates produce excitatory effects that are dissociated from direct dopaminergic actions. An electrophysiological study, by Cayton and Bradley²², of striatal neurones also seems to support the notion that dopamine and morphine (enkephalin) may exert identical effects on the same neurone or population of neurones through different receptor systems. These investigators

reported that both morphine and dopamine inhibited the induced activation of neurones in the caudate nucleus after iontophoretic applications. The dopamine effect was reversed by a specific dopamine antagonist but not by naloxone while the morphine effect was antagonised by naloxone but not by the dopamine antagonist.

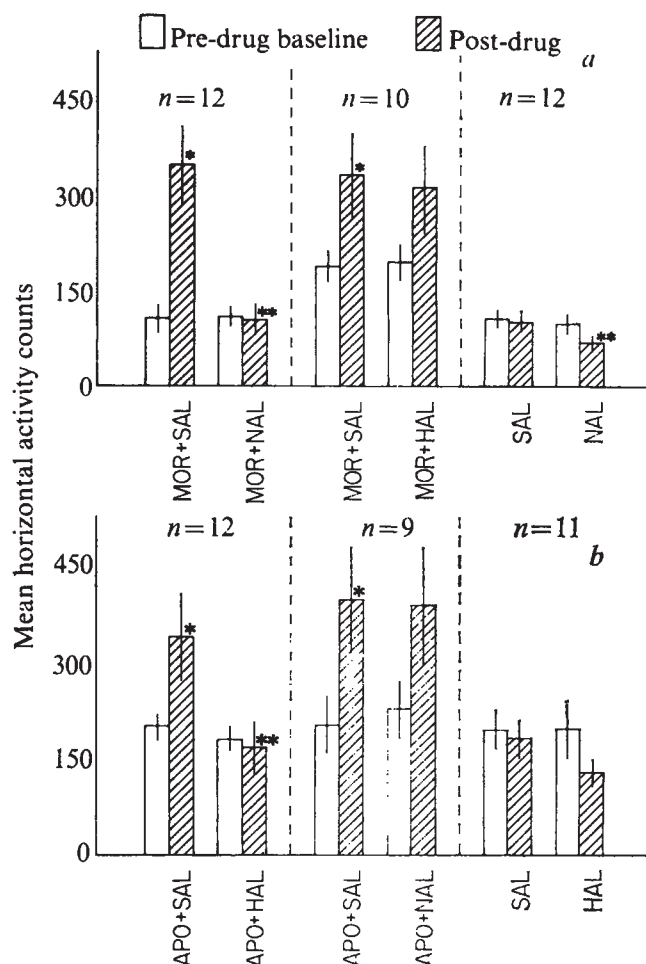


Fig. 2 Effects of naloxone (NAL) 10 mg per kg bodyweight i.p., haloperidol (HAL) 0.25 mg per kg bodyweight i.p. or saline (SAL) 1 ml per kg bodyweight pretreatment on horizontal hypermotility induced by injections of *a*, morphine (MOR), or *b*, apomorphine (APO) in the NA. Right side of figure illustrates the effect of systemically administered NAL (*a*) or HAL (*b*) on spontaneous motility. Similar results were obtained for vertical activity. Some animals were used for more than one drug interaction determination. All drug injections were spaced at least 5 d apart. Baseline activity measures were always determined 1 d before drug injection. Vertical lines indicate s.e.m. * $P < 0.05$ for comparison with baseline activities. ** $P < 0.05$ for comparison of MOR + NAL, and APO + HAL with MOR + SAL and APO + SAL, respectively.

It is possible that parallel neurohumoral systems exist in the forebrain to regulate motility—one encoded by dopamine and the other by enkephalin (the endogenous opiate neuromodulator or neurotransmitter)*.

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Decreased foetal amino acid uptake, brain pyruvate kinase and intrauterine damage in maternal PKU

THE mammalian placenta can concentrate amino acids and release them into the foetal circulation, where they appear at levels higher than on the maternal side¹. This mechanism provides a larger pool for foetal protein synthesis, but normal placental function can become a handicap in some situations. In the presence of an excess of amino acids the placenta exacerbates the maternal imbalance and produces a prolonged hyperaminoacidaemia in the foetus². This in turn may affect the transplacental transport of other amino acids. Such can be the case in 'maternal phenylketonuria (PKU)', which often results in offspring with brain damage

Pregnant rats from a homogeneous colony (CFN, Carworth) were fed either a commercial regular ration (C), or the same diet plus 7% L-phenylalanine (Phe) during the last week of gestation (HiPhe). Placental transport was investigated on the day before delivery. One hour before the procedure, HiPhe animals were given an oral bolus of Phe (500 mg kg⁻¹) to ensure consistent hyperphenylalaninaemia throughout the ensuing procedure, comparable with the situation of an untreated case of human PKU. In separate studies we determined that rats limited to the diet with 7% Phe would have 600–1,000 µM Phe in the blood. After anaesthesia with urethane (1.5 g kg⁻¹), rats were injected in the femoral vein with tracer amounts of metabolites tagged with tritium at 40 µCi kg⁻¹, as described³ and applied⁴ before. The identity of the label was checked by thin-layer chromatography and liquid scintillation counting. The brains of some foetuses in each litter were removed immediately and prepared for enzyme assays^{5,6}. Free amino acid concentrations were determined in blood and tissues^{7,8}.

C foetuses had higher concentrations than the corresponding dams for all three aromatic amino acids tested. Foetuses of HiPhe rats had even higher blood Phe than the dams (Table 1). Blood Tyr concentrations were also higher in HiPhe animals. This is a well known feature of this animal model, because high Phe intake inhibits Phe hydroxylase only partially and does not prevent increased formation of Tyr from its precursor, Phe. If the rate of Tyr transport had been similar in both groups of rats, the concentrations on the foetal side in HiPhe foetuses would have exceeded the solubility of Tyr, approximately 3 mM in water at physiological temperatures. Although there were no differences in blood Trp levels in dams fed either one of the two regimes, the foetuses of hyperphenylalaninaemic

Table 1 Maternal and foetal blood and brain amino acid concentrations; radioactivity after amino acid pulse

Group	Units	Phenylalanine			Tyrosine			Tryptophan		
		Maternal blood	Foetal blood	Foetal brain	Maternal blood	Foetal blood	Foetal brain	Maternal blood	Foetal blood	Foetal brain
Controls	µM	165 ± 41	315 ± 67	355 ± 48	65 ± 11	365 ± 26	798 ± 102	30 ± 6	106 ± 2	126 ± 13
HiPhe	µM	3,127 ± 491‡	4,770 ± 818	2,484 ± 241‡	528 ± 99‡	798 ± 138*	936 ± 62	34 ± 4	48 ± 9‡	68 ± 6‡
Controls	d.p.m. g ⁻¹	—	—	—	66,438 ± 7,159	51,694 ± 4,337	44,381 ± 1,320	38,278 ± 3,390	50,883 ± 4,131	40,735 ± 4,392
HiPhe	d.p.m. g ⁻¹	—	—	—	66,027 ± 6,295	53,972 ± 3,964	37,335 ± 2,222*	36,695 ± 2,268	28,523 ± 2,514‡	16,251 ± 1,090‡

Values represent the means ± s.e. for 7–18 dams and their litters. Specimens were taken 15 min after injection of the tracers. Foetal brains from whole litters were pooled for the assays. Either 3,5-³H-L-tyrosine, 60 Ci mmol⁻¹ or ³H-L-tryptophan, 8 Ci mmol⁻¹, were given at a dose of 40 Ci kg⁻¹.

*P < 0.02 compared with controls.

‡P < 0.01 compared with controls.

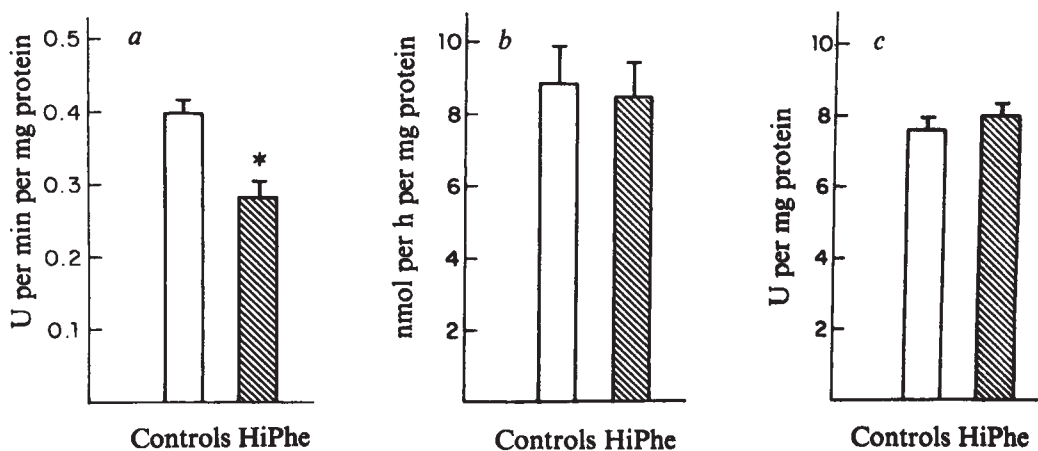
‡‡P < 0.001 compared with controls.

and mental retardation⁹. Using an animal model of 'maternal PKU' we have studied alterations in tissue levels and placental transport of L-tyrosine (Tyr), L-tryptophan (Trp) and certain foetal brain enzymes. We have found a reduced foetal tissue concentration of Trp and impaired foetal brain uptake of Tyr and Trp in the foetuses of hyperphenylalaninaemic dams, as well as reduced brain pyruvate kinase activity.

mothers had very significantly lower blood and brain Trp.

Fifteen minutes after pulse of ³H-Tyr, the radioactivity present in maternal and foetal blood was not different in HiPhe and C animals. That reaching the brain, however, was lower in HiPhe foetuses. In contrast, after injection of ³H-Trp, while counts in maternal blood were also similar in the two groups, foetal blood and brain radioactivity was significantly lower in the HiPhe group.

Fig. 1 Foetal rat brain enzymes from C and HiPhe dams. Pyruvate kinase (a) and hexokinase (b) were determined by the methods of Weber⁵ and succinic dehydrogenase (c) by the procedure of Adlard and Dobbing⁶. Eight pooled litters were assayed in each group. T, means ± s.e.m. *, P < 0.01.



Among the brain enzymes studied, we found that pyruvate kinase was inhibited in HiPhe fetuses, while hexokinase and succinic dehydrogenase were not affected (Fig. 1). These results agree with the findings of several investigators^{8,9,10} who showed *in vitro* and *in vivo* inhibition of rat and human brain pyruvate kinase by Phe. Their results yielded a K_i of of 3–5 mM on rat brain pyruvate kinase. Since the mean brain Phe concentration in the HiPhe fetuses was almost 2.5 mM (compare Table 1), seven times higher than in C, and of the same order of magnitude as the reported K_i the occurrence of this effect as a consequence of transplacental overflow of Phe was well justified.

Placental transport of Trp was investigated because the active transport of this amino acid may be inhibited competitively by Phe. It has been postulated that both share an active translocation mechanism¹¹. The relevance of Trp to the problem of PKU is also tied to the defect in the metabolism of a Trp derivative, serotonin, considered to occur in that condition¹². The synthesis of brain serotonin is regulated both by the availability of Trp and its hydroxylation by Trp hydroxylase, the rate-limiting enzymatic step^{13,14}. In the animal model used here we have observed that the concentration of Trp in the brain of HiPhe fetuses is sharply decreased and that the transplacental passage of tritiated Trp is also lower in HiPhe dams, indicating that substrate availability is reduced in maternal hyperphenylalaninaemia. Moreover, it has been shown¹⁵ that Phe at 10^{-4} to 10^{-5} M is inhibitory of Trp hydroxylase and Trp uptake by a brain stem synaptosome fraction. Thus, the findings on amino acid concentrations in foetal blood and brain suggest that fetuses exposed to the experimental conditions are at a disadvantage on two accounts for normal brain serotonin synthesis.

In previous studies we observed that hyperphenylalaninaemia was associated with an impaired transport across the small intestine of Phe and Trp, but not of Tyr¹⁶. A reduction of blood Phe levels was followed by a normalisation of intestinal absorption. In the present investigation we identified another biological membrane where the interaction between Phe and other aromatic amino acids can result in a severe impairment of the transport of Trp, and of Tyr, to a lesser extent. The resulting reduction in foetal blood and brain Trp, as well as the accumulation of Phe in foetal tissues, can provide a better understanding of the biochemical factors likely to be responsible for the *in utero* damage suffered by fetuses of hyperphenylalaninaemic mothers. Increasing foetal availability of Trp by dietary manipulations¹⁷ may have a possible impact of equal magnitude as decreasing maternal blood Phe, a therapeutic approach already suggested in the prevention of otherwise irreversible damage occurring in human 'maternal PKU'^{13,18}.

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Physiological significance of α -adrenoceptor-mediated negative feedback mechanism regulating noradrenaline release during nerve stimulation

NORADRENERGIC nerve endings in the peripheral nervous system are involved in the synthesis, storage, release and uptake of the neurotransmitter. The view that released noradrenaline interacts only with the postsynaptic receptors of the effector cell to elicit the typical response (contraction of a smooth muscle, positive chronotropic and inotropic effects) has been challenged by the proposal that specific receptors might also be present in the outer surface of the membrane of nerve endings^{1–3}. These presynaptic receptors are of the α -adrenergic type, mediating a negative feedback mechanism regulating noradrenaline release during nerve stimulation^{1–3}. The noradrenaline thus released, on reaching a threshold concentration in the synaptic gap, activates presynaptic α adrenoceptors triggering a negative feedback mechanism that inhibits further release of the transmitter. In support of this view, it has been reported that activation of presynaptic α adrenoceptors leads to a decrease in transmitter release, while blockade of these receptors results in an increase in noradrenaline release during nerve stimulation⁵. We have now demonstrated that the negative feedback mechanism mediated by presynaptic α adrenoceptors has a physiological role in neurotransmission.

In tissues where the effector organ's responses are mediated by α adrenoceptors, block of the presynaptic α receptors enhances noradrenaline release by nerve stimulation, but decreases the responses by blockade of the postsynaptic receptors^{2,6}. On the other hand, for tissues in which the responses are mediated by postsynaptic β adrenoceptors, block of presynaptic α receptors should increase noradrenaline release and the responses to sympathetic nerve stimulation. Nevertheless, in the perfused cat heart⁷ and in isolated guinea pig atria⁸ there are no significant increases in the positive chronotropic responses to nerve stimulation in conditions in which the release of noradrenaline was increased by phentolamine.

An enhancement in transmitter release which is not accompanied by an increase in the response of the effector organ might indicate that the negative feedback mechanism mediated by presynaptic α adrenoceptors does not play a physiological role in noradrenergic neurotransmission. To explore this possibility further, the responses to accelerans nerve stimulation and the increase in transmitter release in isolated guinea pig atria were investigated during and after exposure to the α -receptor blocking agent, phentolamine.

Guinea pig atria with their accelerans nerve, in an isolated organ bath fitted with platinum electrodes for nerve stimulation⁹, were immersed in Locke's solution with $1.4 \mu\text{M}$ atropine. The spontaneous contractions of the preparation were recorded with a force displacement transducer connected to a Grass polygraph. The endogenous noradrenaline stores were labelled with $(\pm)$ -7-³H-noradrenaline⁹ and the accelerans nerves were stimulated with square pulses of 0.5 ms duration and supramaximal voltage.

The overflow of labelled transmitter elicited by nerve stimulation was calculated as fractional release per shock :

Table 1 Effects of phentolamine on ^3H -noradrenaline release elicited by nerve stimulation in guinea pig atria

Experimental group	n	Fractional release per shock ($\times 10^{-4}$)			Ratio	
		S_1	S_2	S_3	S_2/S_1	S_3/S_1
Control	6	1.11 ± 0.18	1.12 ± 0.22	0.89 ± 0.17	1.03 ± 0.11	0.80 ± 0.09
Phentolamine $0.31 \mu\text{M}$	6	1.38 ± 0.28	$4.42 \pm 0.55^\dagger$	$2.33 \pm 0.30^*$	$3.65 \pm 0.62^*$	$1.92 \pm 0.30^*$
Phentolamine $3.1 \mu\text{M}$	6	1.23 ± 0.14	$6.67 \pm 1.65^\ddagger$	$4.39 \pm 0.76^\ddagger$	$6.09 \pm 0.73^\ddagger$	$3.51 \pm 0.30^\ddagger$

Fractional release per shock is expressed as total nCi released per shock divided by the total nCi remaining in the tissue at the onset of nerve stimulation (4 Hz, during 60 s, with supramaximal voltage). Phentolamine (0.31 or $3.1 \mu\text{M}$) was added to the medium 30 min before S_2 and washed out 5 min after S_2 . The interval between each period of nerve stimulation was 50 min. Mean values $\pm$ s.e.m. are shown. n, Number of experiments.

* $P < 0.05$ compared with the control.

† $P < 0.025$ compared with control.

‡ $P < 0.005$ compared with control.

total nCi released per shock divided by the total nCi remaining in the tissue at the onset of stimulation¹⁰. Dose-response curves to exogenous noradrenaline were determined cumulatively¹¹.

During exposure to $0.31 \mu\text{M}$ phentolamine there was a nearly fourfold increase in ^3H -transmitter release elicited by nerve stimulation (Table 1). The enhancement in transmitter release was still observed after the α -blocking agent was removed from the perfusion medium (Table 1).

We studied, separately, the positive chronotropic effects of accelerans nerve stimulation at low frequencies. After four control periods of nerve stimulation, $0.31 \mu\text{M}$ phentolamine was added to the medium and a significant increase in the responses to nerve stimulation was obtained (Fig. 1). As observed for the effects of phentolamine on ^3H -transmitter release, the potentiation of the chronotropic responses elicited by nerve stimulation persisted after the α -receptor blocking agent was removed from the perfusing medium (Fig. 1).

The effects of phentolamine on transmitter release were concentration-dependent (Table 1). When the positive chronotropic responses to nerve stimulation were determined in the presence of $3.1 \mu\text{M}$ phentolamine, however, the increase in responses to nerve stimulation was not statistically significant: in the controls the rate was increased by 31 ± 7 beats per min, while in the presence of

$3.1 \mu\text{M}$ phentolamine the increase was 47 ± 8 beats per min ($n=10$, $P>0.05$). The absence of a significant potentiation in the responses to nerve stimulation in the presence of $3.1 \mu\text{M}$ phentolamine seems to be related to the ability of the α -receptor blocking agent to elicit a negative chronotropic effect and to antagonise responses of the atrial pacemaker to noradrenaline. Exposure to phentolamine produced a negative chronotropic effect which was concentration-dependent. The maximum decrease in atrial rate was obtained with $31 \mu\text{M}$ phentolamine: a reduction by 51 ± 4 beats per min (mean $\pm$ s.e.m. of 13 experiments). During exposure to $3.1 \mu\text{M}$ phentolamine the rate decreased by 16 ± 3 beats per min ($n=13$). The negative chronotropic effect of phentolamine seems to be veratramine-like¹² since there was a significant correlation between the initial rate and the magnitude of the negative chronotropic effect of phentolamine: the higher the atrial rate, the more pronounced the negative chronotropic effect ($r=0.708$, $P<0.01$, $n=25$).

Exposure to $31 \mu\text{M}$ phentolamine produced a marked negative chronotropic effect and a shift to the right in the dose-response curve to exogenous noradrenaline (Table 2).

Table 2 Antagonism by phentolamine of the positive chronotropic responses to noradrenaline in isolated guinea pig atria

Experimental group	n	Initial rate* (beats per min)	Responses to (-)-noradrenaline	
			$pD_{25}^\dagger$	Maximum increase‡
Controls	6	209 ± 9	6.82 ± 0.13	119 ± 10
Phentolamine $31 \mu\text{M}$	8	$136 \pm 8^\S$	$5.54 \pm 0.27^\S$	135 ± 7

* Rate before the beginning of the concentration effect curve to exogenous noradrenaline.

† Mean negative log molar ED_{50} .

‡ Maximum increase in atrial rate (in beats min^{-1}) in response to (-)-noradrenaline.

Mean values $\pm$ s.e.m. are shown. n, number of experiments.

§ $P < 0.05$ compared with control.

|| $P < 0.001$ compared with control.

Phentolamine ($3.1 \mu\text{M}$) also reduced the positive chronotropic responses to noradrenaline. The positive chronotropic effects of 6 and 18 nM noradrenaline were 15 ± 2 and 22 ± 4 beats per min, respectively (mean $\pm$ s.e.m. of six observations). In the presence of $3.1 \mu\text{M}$ phentolamine these values were reduced to 4 ± 1 and 10 ± 3 beats per min (mean $\pm$ s.e.m. of seven observations, $P<0.025$ and $P<0.001$, respectively).

The antagonism by phentolamine of responses of the atrial pacemaker to noradrenaline probably explains why the responses to accelerans nerve stimulation were not increased during exposure to $3.1 \mu\text{M}$ α -receptor blocking agent, in spite of the considerable increase in transmitter release (Table 1).

With the concentration of phentolamine lacking negative chronotropic effects ($0.31 \mu\text{M}$), a significant increase in responses to nerve stimulation was obtained (Fig. 1) when

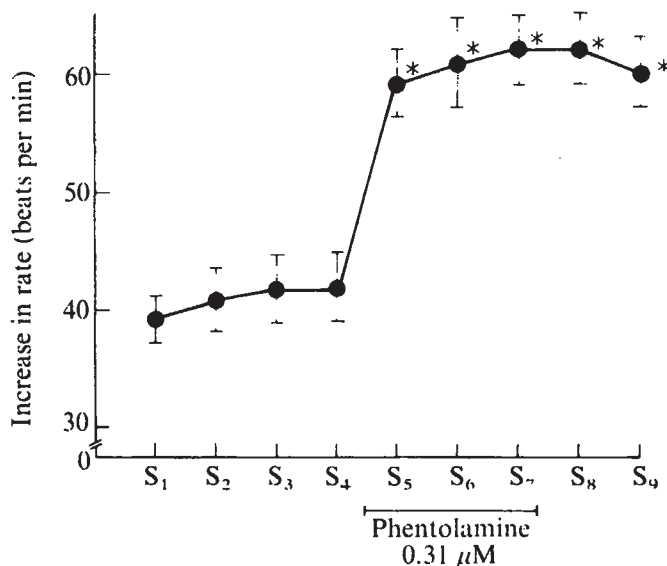


Fig. 1 Potentiation by phentolamine of the positive chronotropic effects of accelerans nerve stimulation in isolated guinea pig atria. Increase in atrial rate induced by nerve stimulations S_1 – S_9 : at 0.5 Hz, for 10 s, supramaximal voltage. The interval between each nerve stimulation was 20 min. Phentolamine ($0.31 \mu\text{M}$) was added to the medium 10 min before S_5 and kept until 10 min after S_8 . In the absence of phentolamine the responses to nerve stimulation were sustained from S_1 to S_9 . Mean values $\pm$ s.e.m. of 11 experiments are shown. * $P < 0.05$ compared with S_1 .

the release of noradrenaline was enhanced (Table 1). This increase in response supports the view that the negative feedback mechanism mediated by presynaptic α -adrenoceptors plays a major physiological role in the regulation of noradrenergic neurotransmission.

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Rapid test to detect agents that damage human DNA

PERHAPS the most successful screening test to detect potential mutagens and/or carcinogens is that of Ames *et al.*¹, using *Salmonella*. Attempts to use mammalian cells have generally involved measuring some cellular response to DNA damage, for example, induction of DNA repair²; if the response implies that an agent has damaged the DNA, the agent is considered to be a potential mutagen and/or carcinogen. An early response to DNA damage in human cells is inhibition of DNA synthesis. But certain non-DNA-damaging agents also inhibit DNA synthesis. I show here that DNA-damaging and non-DNA-damaging agents can be distinguished by the changes in the rates of DNA synthesis after treatment: with a DNA-damaging agent, the rate of DNA synthesis continues to decrease, but with a non-DNA-damaging agent the rate of DNA synthesis increases after treatment.

The test measures thymidine uptake into the DNA of human (HeLa) cells at various times after treatment with a presumptive mutagen or carcinogen. The results with several known mutagenic agents (Fig. 1a-d) show that in every case the amount of thymidine incorporated into the DNA decreased during the first 1-2 h after the end of treatment (after removal of the medium containing the chemical or removal of the cells from the radiation field). Ethyl methanesulphonate (EMS) gave results similar to those with methyl methanesulphonate (MMS), except that 10 mM EMS was required to obtain positive results (data not shown). Benzpyrene requires metabolic activation, and when benzpyrene was incubated with HeLa cells and a microsomal preparation from rat liver, the S-9 mixture³, it was active, whereas alone it was not. In some experiments ³H-leucine was used to measure protein synthesis during the same time that ¹⁴C-thymidine was used to measure DNA synthesis. This is illustrated for 4-nitroquinoline-1-oxide (4NQO) only, but in all cases tested the effect of the agent on protein synthesis was less than the effect on DNA synthesis.

Agents that inhibit DNA synthesis but do not damage DNA—cycloheximide, hydroxyurea, dimethyl sulphoxide, and NiCl₂—were also tested (Fig. 2). In contrast to the results with DNA-damaging agents, the rate of thymidine

incorporation into DNA increased rapidly immediately after the removal of these agents from the medium (although complete recovery sometimes took a few hours). Phorbol myristate acetate, a so-called promoter of carcinogenesis, was also found in this class (data not shown).

We explain this difference between the two classes of agents that inhibit DNA synthesis as follows. When an agent that inhibits by metabolic means is removed from the medium, the metabolic block is removed, recovery begins immediately, and the rate of DNA synthesis increases. When a DNA-damaging agent is removed from the medium,

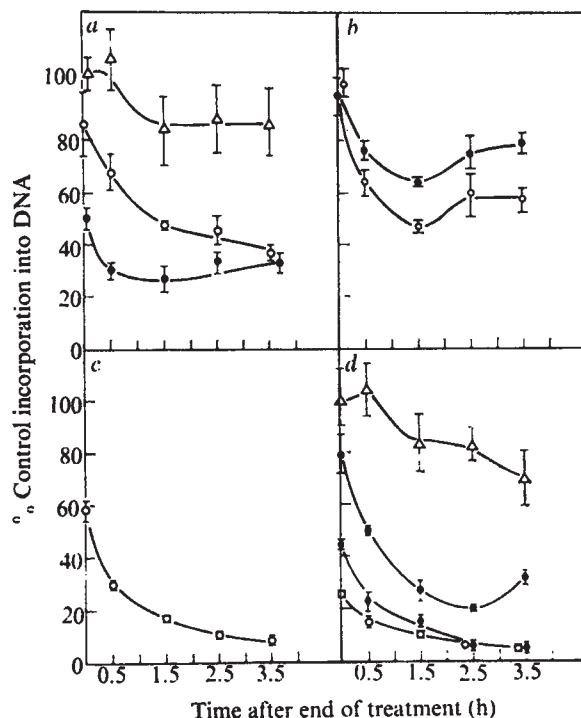


Fig. 1 Rate of DNA synthesis, measured as percentage of control incorporation of thymidine into DNA, in HeLa cells at various times after treatment with a, benzpyrene; b, MMS; c, ultraviolet light (10 J m^{-2}); d, 4NQO. a, Δ , $7 \mu\text{M}$; $\circ$, $7 \mu\text{M}$ + S-9; $\bullet$, $20 \mu\text{M}$ + S-9. b, $\bullet$, 0.5 mM ; $\circ$, 1 mM . d, Δ , $0.2 \mu\text{M}$ (protein synthesis); $\bullet$, $0.2 \mu\text{M}$; $\diamond$, $1 \mu\text{M}$; $\circ$, $2 \mu\text{M}$. HeLa cells were grown in Eagle's minimal essential medium (MEM) with 15% calf serum. 4NQO was dissolved in alcohol at 0.2 mM . Benzpyrene was dissolved in alcohol at 1 mM and diluted into McCoy's 5a medium containing 2.5% calf serum and a 1/20 dilution of S-9 mix, a microsomal preparation from rat liver prepared from animals previously injected with Aroclor, a polychlorinated biphenyl³. Some cultures were treated with benzpyrene in McCoy's medium without S-9 mix. The S-9 mix was simultaneously tested and found to have no effect on DNA or protein synthesis at the dilutions used. Incubation times were 30 min for MMS and 4NQO and 1 h for benzpyrene. Ultraviolet light irradiations were performed on cells under saline at $1.3 \text{ J m}^{-2} \text{ s}^{-1}$. Immediately after incubation with the chemical or after exposure to ultraviolet light, the medium was removed from all cultures (including controls, which were treated identically except for treatment with the agent to be tested), and Eagle's MEM was placed back on all but two controls and two cultures treated with the agent. These were immediately incubated for 10 min with MEM containing either ¹⁴C-thymidine ($0.3 \mu\text{Ci ml}^{-1}$; 50 mCi mmol^{-1}) and ³H-leucine ($10 \mu\text{Ci ml}^{-1}$; 84 Ci mmol^{-1}) or ³H-thymidine ($10 \mu\text{Ci ml}^{-1}$; 50 Ci mmol^{-1}) alone. (When ³H-thymidine was used, the cells had been incubated for 24 h or more with $0.02 \mu\text{Ci ml}^{-1}$ ¹⁴C-thymidine before treatment with the agent.) At each of the times indicated, two more controls and two more treated cultures were incubated in radioactive medium for 10 min. The cells were washed after each incubation, scraped into SSC (0.15 M sodium chloride; 0.015 M sodium citrate), and filtered through GF/C (Whatman) glass filters moistened with cold 4% perchloric acid. The filters were washed with cold 4% perchloric acid, 70% alcohol, and 100% alcohol, dried, and added to vials containing scintillation fluid; their ¹⁴C and ³H content was measured by liquid scintillation spectrometry. Error bars indicate the range of values obtained in a single experiment.

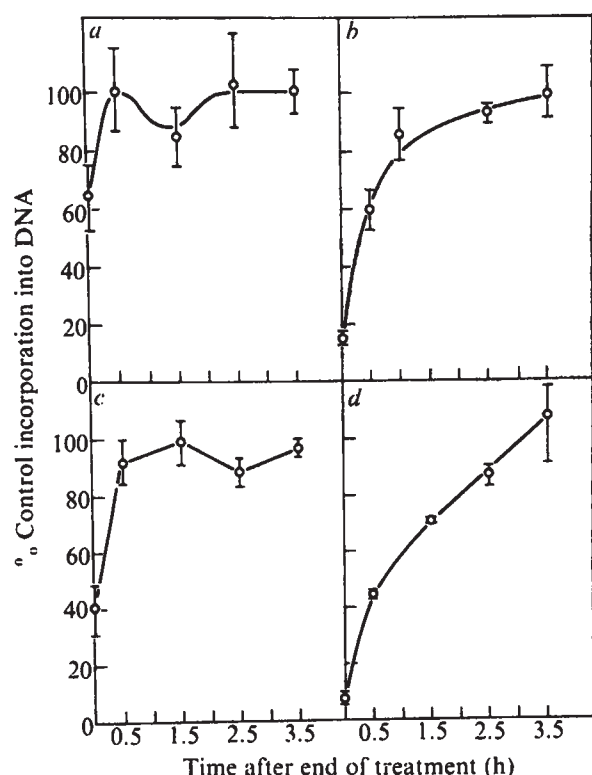


Fig. 2 Rate of DNA synthesis in HeLa cells at various times after treatment with agents that inhibit DNA synthesis by metabolic mechanisms. Conditions were the same as for Fig. 1. a, NiCl_2 (5 mM); b, DMSO (1.3 M); c, cycloheximide (23 μM); d, hydroxyurea (1 mM).

however, the damage remains, and the rate of DNA synthesis decreases with time. This occurs for one or both of two reasons. First, certain agents, such as ultraviolet light, induce lesions that block the progression of growing points in replicating DNA^{4,5} so that progressively fewer growing points remain in operation, until repair occurs. Second, other agents, such as X rays⁶⁻⁸ and MMS⁹, block initiation of replicons, so that as more and more replicons terminate after damage is incurred, DNA synthesis progressively decreases¹⁰.

My results suggest that measurements of DNA synthesis within the first 1–2 h after treatment with an agent can determine its potential as a DNA-damaging agent and therefore as a mutagenic and potentially carcinogenic agent. The simplest procedure is to label HeLa cells for one generation or more (≥ 24 h) with ^{14}C -thymidine before treatment and to pulse with ^3H -thymidine for 10 min at various times after treatment, comparing the resulting $^3\text{H}/^{14}\text{C}$ ratios with those measured at the same times in controls. Although in many of the experiments reported here prelabel was omitted, so that synthesis of both protein (with ^3H -leucine) and DNA (with ^{14}C -thymidine) could be measured after treatment, it is not necessary to measure the former to detect DNA-damaging agents. Furthermore, the use of ^{14}C -thymidine to label parental DNA before treatment with the suspected agent makes possible greater flexibility; for example, care need not be taken to put exactly the same number of cells in each plate, because the resulting $^3\text{H}/^{14}\text{C}$ ratios are independent of cell number.

The experiment using S-9 demonstrates that the method can detect agents requiring metabolic activation. Cyclophosphamide, which also requires activation, required a $2.5\times$ greater concentration of S-9 to achieve activation (data not shown) than did benzpyrene. Thus, each agent needs to be tested for the optimal conditions for activation, as has been reported for other detection systems³.

The results with NiCl_2 require comment. Nickel in the

metallic form has been shown to be carcinogenic in rodents¹¹. Unpublished work in my laboratory, however, has shown that NiCl_2 inhibits DNA synthesis by slowing (not blocking) the rate of chain elongation, in a manner similar to cycloheximide¹² and hydroxyurea¹³, and therefore is presumably not a DNA-damaging agent.

The advantages of my method for detecting DNA-damaging agents are: (1) HeLa cells are of human origin and are probably representative of the reaction of other human cells—presumably other rapidly growing human cells could also be used; (2) HeLa cells are easy to grow and do not exhibit contact inhibition, so that enough cells can easily be obtained in a 35-mm Petri dish or other small test vessel for a single determination; (3) the test requires only about 2 h from the first addition of the agent to the end of the last incubation, and (4) the assay is extremely simple—no extraction procedures are necessary because the whole cells are precipitated on to filters, which are dried and counted by conventional procedures. The disadvantages are similar to those of other systems based on DNA damage, for example the system will not detect mutagens, such as asbestos, that act through unknown mechanisms. False positives do not seem to be a factor. Possibly some agent could act so that metabolism in general, and consequently DNA synthesis, would continue to decrease with time after the agent has been removed from the cell. Experiments with KCN, however, suggest that this is unlikely. KCN at 15 and 30 mM decreased the DNA synthesis rates to about 25 and 15% of the controls, respectively; nevertheless, after KCN was removed, recovery of DNA synthesis began immediately for the lower concentration and within 1.5 h for the higher. Thus, recovery of DNA synthesis occurs from even a powerful general poison, as long as no DNA damage is incurred. It seems possible, therefore, that this simple test might be useful as a screening procedure to detect agents that damage human DNA.

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Functional identity of a mouse ascites and a rabbit reticulocyte initiation factor required for natural mRNA translation

INITIATION of eukaryotic protein synthesis involves the assembly of a functional initiation complex from components including ribosomal subunits, initiator tRNA (Met-tRNA_i), initiation factors, messenger RNA, ATP and GTP¹⁻³. Much research in this area has centred on the possible control of eukaryotic translation at the level of

polypeptide initiation, as this step seems to be rate-limiting in the overall synthetic reaction^{4,5}. Some studies have suggested the existence of tissue-specific initiation factors⁶, others have described initiation factors which apparently discriminate between eukaryotic mRNAs⁷. Work in this laboratory led to the purification of an initiation factor of molecular weight 53,000 from the supernatant fraction of Krebs II ascites cells⁸. This factor (IF_{EMC}) was shown to be required for the translation of encephalomyocarditis virus RNA (EMC RNA) but not for globin mRNA translation in a fractionated ascites cell-free system⁹. Other studies on the translation of mRNAs injected into living *Xenopus laevis* oocytes¹⁰, and on the translation of various mRNAs in several crude eukaryotic protein-synthesising systems¹¹ have suggested, however, that eukaryotic translation proceeds without controls exerted by specific initiation factors. We have adopted a comparative approach to this problem, examining the translation of various eukaryotic mRNAs in a fractionated cell-free system from Krebs II ascites cells, and testing the specificity of two mammalian factors reported to be required for the initiation of natural mRNA translation.

The specificity of the IF_{EMC} factor was investigated further (Table 1). Our results confirm work demonstrating requirement for IF_{EMC} in EMC RNA translation⁹. Translation of mengo virus RNA and foot-and-mouth disease virus RNA was also dependent on the addition of IF_{EMC}. Some mRNAs, however, showed no significant dependence on IF_{EMC} for translation (for example, tobacco mosaic virus RNA), whereas others showed a partial stimulation of translation by IF_{EMC} (for example, Semliki forest virus 26S RNA). Our studies have shown a wide spectrum of dependencies on IF_{EMC} for the translation of several mRNAs, and have demonstrated differences in the efficiency of their translation in the fractionated cell-free system. For example, RNAs from two closely related picornaviruses¹², namely EMC and polio, show differences both in their extent of translation and in their requirement for IF_{EMC}. It is therefore possible that other factors, necessary for translation of mRNAs such as polio RNA, may be either absent or present in suboptimal concentrations in the fractionated cell-free system.

An apparent analogue of Krebs ascites IF_{EMC} termed IF-E4 (ref. 2) or IF-M4 (ref. 13), has been recognised in rabbit reticulocytes, and is similar to IF_{EMC} in molecular weight and chromatographic behaviour. We therefore compared the requirement for IF_{EMC} and IF-E4 in EMC RNA

translation in the IF_{EMC}-deficient ascites cell-free system (Fig. 1). IF_{EMC} and IF-E4 were equally efficient in stimulating EMC RNA translation at saturating factor concentration. The greater specific activity shown by IF-E4 presumably reflects the higher purity of this factor compared with IF_{EMC}.

We then examined separately the specificity of IF-E4 in the translation of several mRNAs in the ascites cell-free system (Table 2). The results correspond closely to those obtained with IF_{EMC} (Table 1). EMC RNA, mengo virus RNA and foot-and-mouth disease virus RNA require the

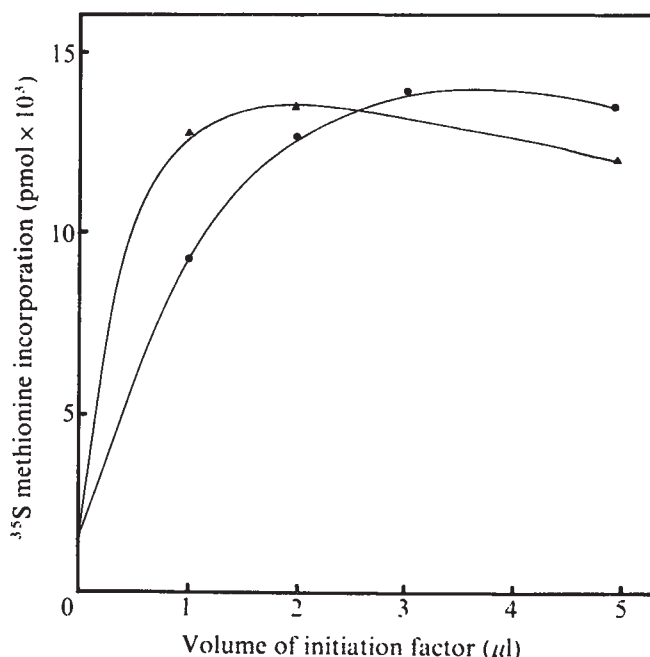


Fig. 1 Dependence of EMC RNA translation in the ascites fractionated cell-free system on IF_{EMC} (from Krebs ascites cells) (●) and IF-E4 (from rabbit reticulocytes) (▲). Protein synthesis assays were performed as described in Table 1, containing 0.02 *A*₂₆₀ units EMC RNA and increasing volumes of IF_{EMC} (protein concentration 1 mg ml⁻¹) or IF-E4 (protein concentration approximately 0.3 mg ml⁻¹). Incorporation in the presence of EMC RNA was corrected for a value of 0.0094 pmol ³⁵S-methionine obtained in the absence of added EMC RNA. No alteration in this background value was observed on addition of either initiation factor. 1 pmol ≈ 26 × 10³ c.p.m.

Table 1 Effect of the addition of Krebs ascites cell IF_{EMC} on the translation of various mRNAs in the fractionated ascites cell-free system

mRNA	³⁵ S-methionine incorporation (pmol × 10 ³)		Stimulation by IF _{EMC} (% control value)
	Control	Control + IF _{EMC}	
Encephalomyocarditis virus	7	55	686
Mengo virus	5	88	1,660
Foot-and-mouth disease virus	18	62	233
Polio virus	11	10	0
Semliki forest virus (42S RNA)	15	12	0
Semliki forest virus (26S RNA)	12	18	50
Vaccinia virus <i>in vitro</i> mRNA	26	27	4
Tobacco mosaic virus	42	44	5
Alfalfa mosaic virus	17	17	0
Brome mosaic virus	11	13	18
Satellite tobacco necrosis virus	34	48	37
Tobacco rattle virus	45	56	24
Haemoglobin mRNA	10	14	40
Oviduct polysomal RNA	12	10	0

The components of the fractionated ascites cell-free system were prepared as previously described⁹. Protein synthesis assays were performed in 25-μl reaction mixtures containing 0.45 *A*₂₆₀ units ascites ribosomes, 5 μg elongation factor I, 0.02 *A*₂₆₀ units ³⁵S-methionine-tRNA (0.8 pmol ³⁵S-methionine), 1–6 μg mRNA, 1–2 μg IF_{EMC}, 20 mM Tris-HCl (pH 7.5), 3.5 mM MgCl₂, 100 mM KCl, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 8 mM creatine phosphate and 100 μg creatine phosphokinase. The ionic conditions were optimal for several mRNAs tested, that is, EMC RNA, Semliki forest virus 26S RNA and tobacco mosaic virus RNA. IF_{EMC} (purified to the Sephadex G-100 stage⁹) and each mRNA was added in saturating concentration. Incubation was at 37 °C for 30 min. Hot trichloroacetic acid-precipitable radioactivity was measured as previously described⁹. Incorporations in the presence of mRNA were corrected for background incorporation of 0.010 pmol ³⁵S-methionine in the absence of added mRNA. 1 pmol ³⁵S-methionine is approximately equivalent to 25 × 10³ c.p.m.

Table 2 Effect of the addition of rabbit reticulocyte IF-E4 on the translation of various mRNAs in the fractionated ascites cell-free system

mRNA	³⁵ S-methionine incorporation (pmol × 10 ³)		Stimulation by IF-E4 (% control value)
	Control	Control × IF-E4	
Encephalomyocarditis virus	28	142	410
Mengo virus	28	159	470
Foot-and-mouth disease virus	51	222	335
Polio virus	48	52	8
Vaccinia virus <i>in vitro</i> mRNA	46	48	4
Tobacco mosaic virus	178	179	0
Brome mosaic virus	62	80	3
Tobacco rattle virus	168	177	5
Satellite tobacco necrosis virus	111	126	14
Haemoglobin mRNA	14	22	57
Oviduct polysomal RNA	39	52	33

Protein synthesis directed by each mRNA was assayed at saturating mRNA concentration as described in Table 1 and the effect of the addition of a saturating amount of IF-E4 was tested. Incorporations in the presence of mRNA were corrected for background incorporation of 0.015 pmol ³⁵S-methionine in the absence of added mRNA. 1 pmol ³⁵S-methionine is $\approx 19 \times 10^4$ c.p.m.

addition of IF-E4 for translation, whereas the remainder of the mRNAs tested show either no significant dependence (for example, tobacco mosaic virus RNA) or a partial dependence (for example, oviduct polysomal RNA) on IF-E4 for translation. The optimal concentration of monovalent and divalent cations for EMC RNA translation in the presence of either IF_{EMC} or IF-E4 was tested and found to be the same for either factor, that is, optimal translation was obtained at 100 mM KCl and 3.5 mM MgCl₂. In common with IF_{EMC}, IF-E4 is required for the synthesis of EMC-specific initiation peptides formed in the presence of sparsomycin¹⁴, an inhibitor of polypeptide elongation, suggesting that both factors act at the level of initiation of protein synthesis.

Staehelein's group has shown that IF-E4 is required for the translation of both globin mRNA and EMC RNA in a completely fractionated protein-synthesising system². Moreover, a twofold higher concentration of IF-E4 was required for optimal EMC RNA translation than for globin mRNA translation. Because EMC RNA is larger than globin mRNA, in molar terms this represents a greater IF-E4 requirement for EMC RNA translation, suggesting that the amount of IF-E4 required for optimal translation varies according to the mRNA tested. Our results are consistent with these findings. The high requirement for IF_{EMC} in EMC RNA translation is expressed by the need to add exogenous factor to the fractionated ascites cell-free system for translation to proceed. A low level of IF_{EMC} contamination of the ascites cell-free system could explain the absence of dependence or partial dependence on IF_{EMC} for the translation of several mRNAs (Table 1). In support of this idea, we have detected IF_{EMC} activity in a 350 mM KCl wash of the ribosomes used in the standard protein synthesis assay. We were unable to prepare ribosomes devoid of IF_{EMC} which retained good activity in natural mRNA translation in the fractionated cell-free system.

Hellerman and Shafritz have suggested that certain initiation factors bind to mRNA¹⁵. Thus both the Met-tRNA_F binding factor and messenger ribonucleoproteins (mRNPs) from rabbit reticulocytes bound to globin mRNA and poly(A). A polypeptide of molecular weight 52,000 was found both in the initiation factor and the mRNP preparation. In spite of the close similarity in molecular weight of this polypeptide to IF_{EMC}, we could not demonstrate co-electrophoresis in sodium dodecylsulphate (SDS) gels of IF_{EMC} with any of the proteins of ascites mRNPs. This suggests that IF_{EMC} does not form a stable association with mRNA.

We conclude that IF_{EMC} and IF-E4 are functionally identical and have analogous roles in the initiation of protein synthesis in rabbit reticulocytes and mouse ascites cells. This factor is therefore not specific to cell, tissue or species but may be required in variable amounts for the translation

of all mRNAs. It is interesting that variable amounts of the prokaryotic initiation factor IF3 are needed for the translation of different bacterial mRNAs¹⁶. IF3 may stabilise the direct base-pairing between the 5' terminus of mRNA and the 3' end of 16S ribosomal RNA shown to be important in the initiation of prokaryotic protein synthesis¹⁷. We speculate that IF_{EMC} and IF-E4 effect a similar stabilisation in eukaryotic mRNA-ribosome interaction¹⁸.

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A search for *in vivo* factors in regulation of microtubule assembly

MICROTUBULES are abundant in eukaryotic cells where they assume various functions¹, some of which seem to be at a purely structural level, such as maintenance of cell shape and axonal elongation, whereas others are involved in more dynamic processes, such as mitosis, meiosis, secretion and axonal transport. At least two distinct protein factors of

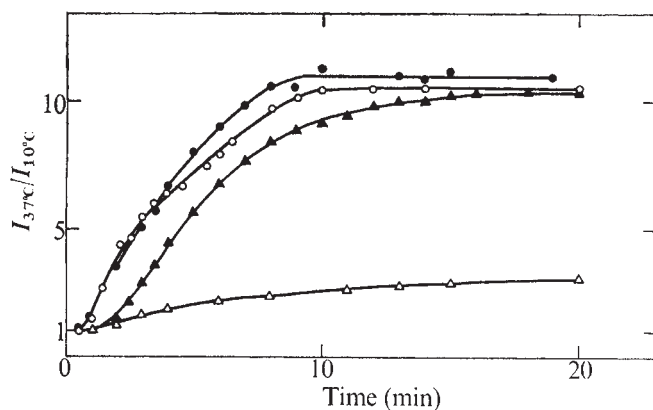


Fig. 1 For preparation of microtubules, rat brains were homogenised in 2 volumes of MES buffer and microtubules polymerised from the 105,000g supernatant using the method of Shelanski *et al.*¹¹. The pellets obtained after centrifugation for 30 min at 50,000 r.p.m. and 25 °C, in a Spinco 50 rotor, were washed, and after resuspension, depolymerised for 30 min at 0 °C. The supernatant obtained after 30 min of centrifugation at 40,000 r.p.m. and 0 °C, in a Beckman 50 rotor, was used to analyse the kinetics of microtubule selfassembly by light scattering, electron microscopy and analytical ultracentrifugation. The rate of polymerisation of brain homogenates was followed by monitoring the time dependence of their scattering at 37 °C at an angle of 90° in a Fica light scattering photometer. The ratio of scattering at 37 °C to that at 10 °C (I_{37}/I_{10}), reflecting the polymerisation process was measured in the absence (open symbols) and presence of glycerol (solid symbols) on brain homogenates from 5-d-old (Δ) and 30-d-old rats ($\circ$). Similar scattering patterns were observed for solutions obtained after a one-cycle polymerisation purification. At 10 °C no change in scattering of solutions was detected during the experiments.

different molecular weight have been suggested²⁻⁵ to regulate microtubule assembly. The uniqueness of their role is not unequivocally established because microtubules have also been obtained in the absence of these protein factors, at high magnesium ion concentrations⁶, in the presence of polycations⁷ or glycerol¹¹. To correlate better the results obtained *in vitro* with the mechanism operating *in vivo*, we have studied the assembly of microtubules in extracts prepared from developing rat brain, differentiating mouse neuroblastoma cells and in chicken sensory ganglia, which may facilitate detection of a factor which induces self-assembly *in vivo*. We conclude that the high molecular weight protein is seemingly not required for the *in vivo* assembly of microtubules.

The rate and extent of polymerisation of microtubules, as measured by light scattering, in soluble extracts prepared from brains of 5-d-old rats was much lower than in extracts from 30-d-old rat brains (Fig. 1). This confirms observations made by Fellous *et al.*⁹⁻¹⁰. Glycerol promotes

tubulin assembly¹¹, and in the presence of glycerol the polymerisation rate of the extract of 5-d-old brain was considerably increased, reaching a level comparable with that of the 30-d-old extract. Depolymerised microtubules prepared from 5-d-old rat brains did not show the ring component in the analytical centrifuge (36S) that was present in extracts from adult animals (Fig. 2). Electron micrographs of these extracts confirm that rings were present only in the 30-d extract. Our measurements of the 'ring' peak indicate a sedimentation coefficient of 30-33S. Thus, as our preparation procedures were similar to those of Shelanski *et al.*¹¹, we conclude that we are dealing with the native tubulin ring sedimenting at 36S, rather than the 42S ring component whose formation was induced by a high magnesium level (see ref. 8, page 4565).

These data raise two main questions: are the microtubules formed in the presence of glycerol similar to those polymerised in its absence, and is there a correlation between the higher rate of polymerisation of microtubules in extracts from mature brains and the presence of 'rings' and other protein species absent from extracts of younger animals? We have observed that microtubules polymerised in the presence or absence of glycerol have the same gross morphology and optical diffraction patterns of their micrographs display identical periodicities. Thus with respect to periodic features the two species are indistinguishable. It should be noted, however, that microtubules polymerised in the presence of glycerol have been reported to contain different amounts of accessory proteins. The presence of glycerol also leads to the formation of rings of apparently different shape or subunit composition, as reflected by their sedimentation coefficients¹².

Rings cannot be formed from purified 6S tubulin, but they can be assembled in the presence of a high molecular weight protein which has yet to be fully characterised³⁻⁵ or in the presence of high magnesium concentration⁸, albeit with apparent change in their structure in the latter case. To test for the presence of this protein factor, extracts were analysed by electrophoresis on SDS-polyacrylamide gels (Fig. 3). There was no clear correlation between the relative content of high molecular weight protein in the extract and the speed of polymerisation, and the relative amount of this protein in microtubules did not correlate with the animal's age. These results suggest that the high molecular weight protein, although able to induce *in vitro* microtubule assembly, is not required for *in vivo* polymerisation. In line with this conclusion is our inability to detect high molecular weight protein by autoradiography of sodium dodecyl sulphate (SDS)-polyacrylamide gels of microtubules from differentiating neuroblastoma cells¹³ or sensory ganglia exposed to nerve growth factor (unpublished observations by H. Schmitt and S. Mizel), labelled with ³H-leucine or ³⁵S-methionine.

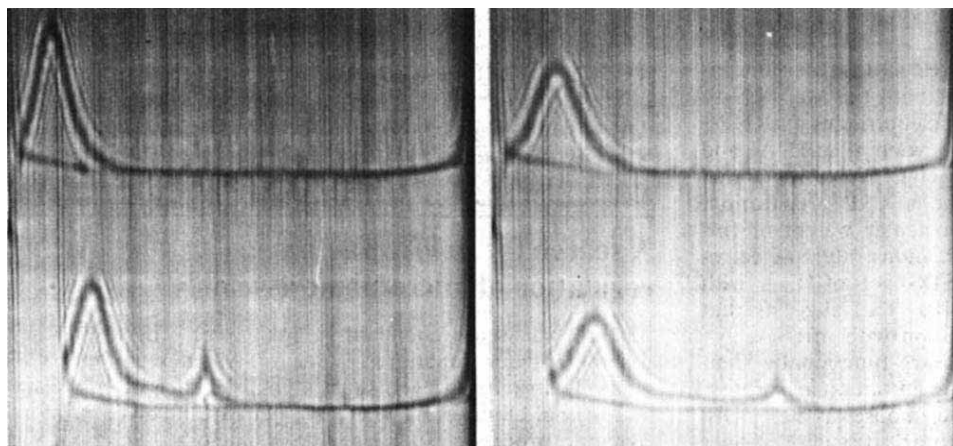


Fig. 2 Schlieren pattern of tubulin sedimenting in the ultracentrifuge at 44,000 r.p.m. and 7 °C. The upper cell contained tubulin from 5-d-old rat brain, and the lower cell, tubulin from 30-d-old rat brain. The first pattern was obtained after 16 min and the second pattern after 32 min of sedimentation. The upper pattern has only a single 6S peak and the lower cell has a 6S and a 36S peak. Electron microscopy confirms that the 36S component consists of rings.

On the other hand, a protein with a molecular weight, on SDS-polyacrylamide gels of 82,000 was reproducibly found in microtubules prepared from brains of rats older than 10 d and not from younger animals (Fig. 3). The molecular weight of this protein is similar to that of τ factor, first described by Weingarten *et al.*^{5,14,15}. This observation supports the report by Fellous *et al.*¹⁰ that partially purified τ factor enhances the rate of polymerisation of microtubules from extracts of young rat brains and raises the possibility that it interacts with tubulin and facilitates polymerisation. A more definitive indication of the role of this protein will be obtained if it can be shown to be associated with microtubules polymerising *in vivo*.

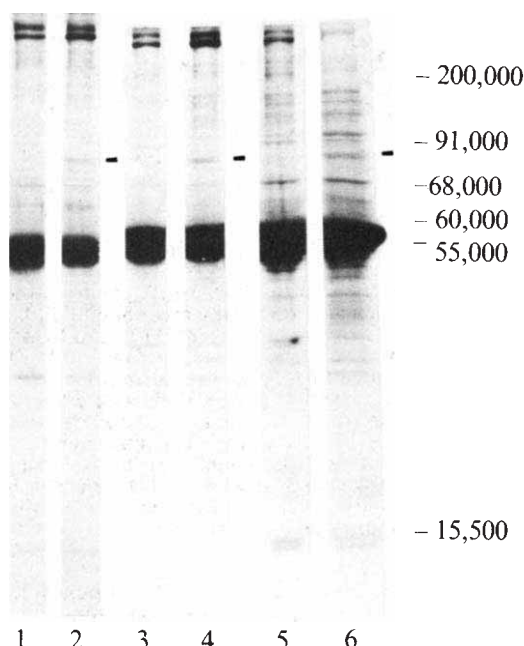


Fig. 3 SDS-polyacrylamide gel analysis of polymerised microtubules from 5- and 30-d-old rat brains. Microtubules were purified by one-cycle of self-assembly as described in Fig. 1. Pellets were resuspended and analysed on 10–20% polyacrylamide slab gels containing 0.1% SDS, as previously described¹³. Markers used to calibrate the gels included myosin (200,000), phosphorylase (91,000), bovine serum albumin (68,000), catalase (60,000), tubulin (55,000) and globin (15,500). 1, 3 and 5 are microtubules purified from 5-d-old rat brains and 2, 4 and 6 are from 30-d-old rat brains.

Our finding that extracts from young rat brains which do not display ring formations polymerise in the presence of glycerol, to yield microtubules similar to those of adult rat brain extracts in which the rings are abundant raises questions as to the validity of extrapolation of *in vitro* studies to the *in vivo* mechanism. Involvement of a membrane-bound initiator protein, with a function similar to that of α -actinin in actin polymerisation, cannot yet be discounted. Such a mechanism could explain the exclusive localisation of microtubules in the growing neurites of the nerve cell.

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Tubulin hoops

CYTOPLASMIC tubulin displays a wide range of polymorphism, including small sheets¹ and rings or spirals². We report here several new structures. One of the most interesting aggregates is a hoop which has a triplet substructure and arrangement of the tubulin dimer, similar to that found in flagellar B tubules.

Tubulin hoops were formed by polymerisation of purified protein in the conditions listed in the legend to Fig. 1. Initially, the preparation contained narrow, long, wavy sheets (about 3–7 protofilaments wide). If allowed to polymerise further, the sheets became wider (between 10 and 15 protofilaments) and straightened out. They sometimes folded into microtubules (13 protofilaments) or associated laterally with other sheets oppositely oriented; other sheets folded into small tubules (8–11 protofilaments). Narrow sheets often curled into hoops of 1,000–1,400 nm in circumference. The hoops are thus shallow spirals, and these grew up to widths of 400 nm (about 80 protofilaments). Depending on how they settled down on the carbon film, different images were obtained (Figs 1, 2).

The outside of the protofilaments in the hoops corresponds to the inside of the protofilaments in microtubules. This is evident from Fig. 2b, where the inner portions of a hoop lie flat on the carbon film, but adjacent stretches do not fold over properly, as in Fig. 2a. The resulting stress leads to breaks or distortions at the corners on the inner side, while the outer rim stays elevated, as verified using platinum shadowed specimens. These images indicate that the outside of the hoop lies on the grid; and by correlating the observed striations with those of the left-handed microtubule helix³, we deduce that the protofilaments in the hoop are turned inside out relative to the microtubule.

Optical diffraction patterns of the hoop images are best understood in terms of the surface lattice of microtubules⁴ or sheets¹. In Fig. 3a the lines connecting the lattice points apply to a microtubule viewed from the front; the back would have the same spacings, but opposite inclinations. The diffraction spots expected from this lattice are asymmetric with respect to the vertical axis if we see either front or back (for example, in sheets, or hoops opened up on the microscope grid (Fig. 2a)), and symmetric when front and back are superimposed, such as in microtubules or hoops viewed from the side (Fig. 1).

Some immediate inferences from optical diffraction patterns are that the hoop side view (Fig. 1) is a two-layered structure showing front and back (spots symmetric about the meridian), that the top views are one layered (asymmetric spots), and that adjacent stretches in a proper top view face opposite ways (Fig. 2a), but in the same direction in a partial top view (Fig. 2b). The spacing of the first layer line is highly reproducible (within 1%), $c=4$ nm in all our polymorphic aggregates, showing the protofilaments to be connected longitudinally along nearly invariant bonds⁵. The lateral separation between protofilaments varies in the hoops but averages 4.7 nm; this corresponds to the separation of the protofilaments near the

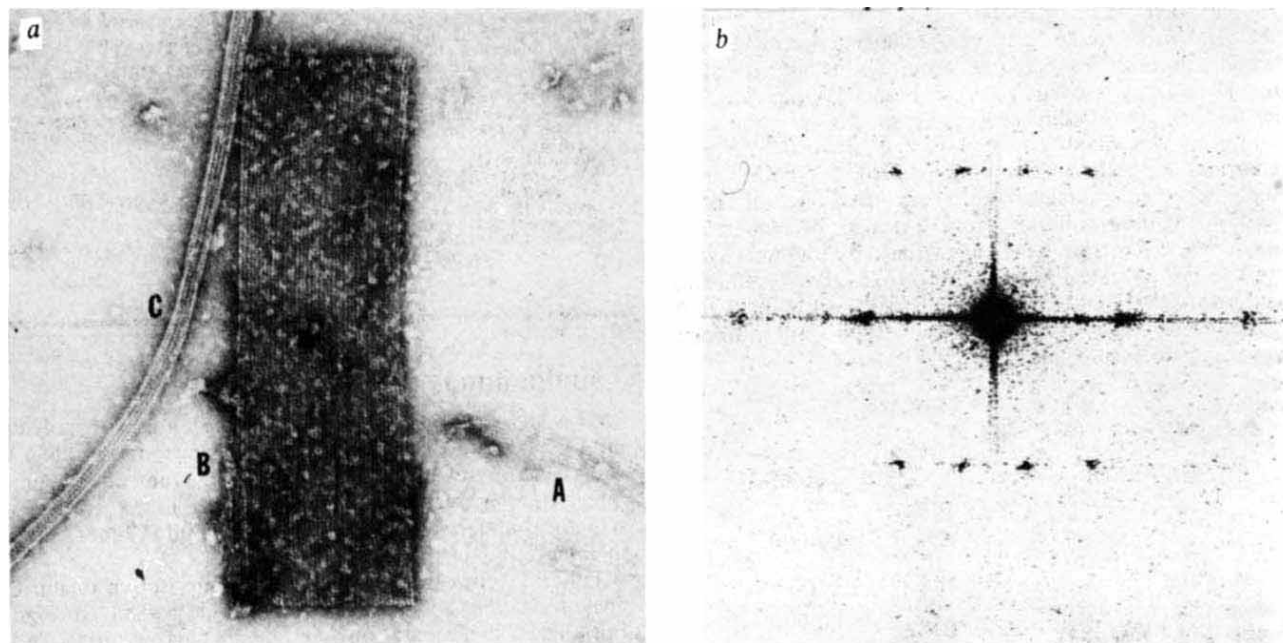


Fig. 1 *a*, Hoop viewed from the side, containing about 40 protofilaments. The probable parent sheet is seen at A, the growth point protruding from the hoop at B. C is a minitubule made from nine protofilaments ($\times 140,000$) Tubulin, prepared essentially according to Shelanski *et al.*¹¹, was purified by three cycles of polymerisation in reassembly buffer (0.1 M MES, 1 mM EGTA, 1 mM GTP, 1 mM DTT, 0.5 mM MgCl_2 , pH 6.5 at 4 °C) with the addition of 25% glycerol. The protein (5–10 mg ml⁻¹ in cold reassembly buffer), was diluted to 1 mg ml⁻¹ or less, by adding warm (25 °C, pH 6.5, 10–25% glycerol) buffer. After 1–2 min the temperature was raised to 37 °C and polymerisation followed for up to 1 h. Samples for electron microscopy were removed at various intervals, placed on carbon support grids, stained with 1% uranyl-formate, and examined in a Philips EM 301 electron microscope. Conditions for minimising electron dose were as described elsewhere¹². *b*, Diffraction pattern of (a). The strong spots on the equator (using the notation of Fig. 3) are the (13, 1) at a spacing of 4.8 nm and (26, 1) at 2.4 nm. Ghost reflections arising from tripling occur at orders of 14.2 nm out to a resolution of 2 nm. The 4-nm layer line is dominated by 4 spots symmetric about the meridian, pairs of (–3, 1) and (10, 1) reflections from front and back, with weak ghosts in between. The 2-nm layer line shows a pair of spots, index (7, 2). Very weak reflections on the 8-nm layer line lie midway between the origin and the (–3, 1) spots. This shows that the arrangement of dimers in the hoops is similar to that in flagellar B subfibres (see text).

centre of the microtubule wall⁶. The shift between adjacent protofilaments also varies and is usually greater than 0.9 nm. Thus, in most cases, 13 adjacent protofilaments cannot be wrapped into a closed microtubule, as is possible

with Erickson's sheets¹. The surface lattice does, however, often fit one of the minitubules mentioned above.

A striking aspect of the images is the periodic tripling of protofilaments. Correspondingly, optical diffraction

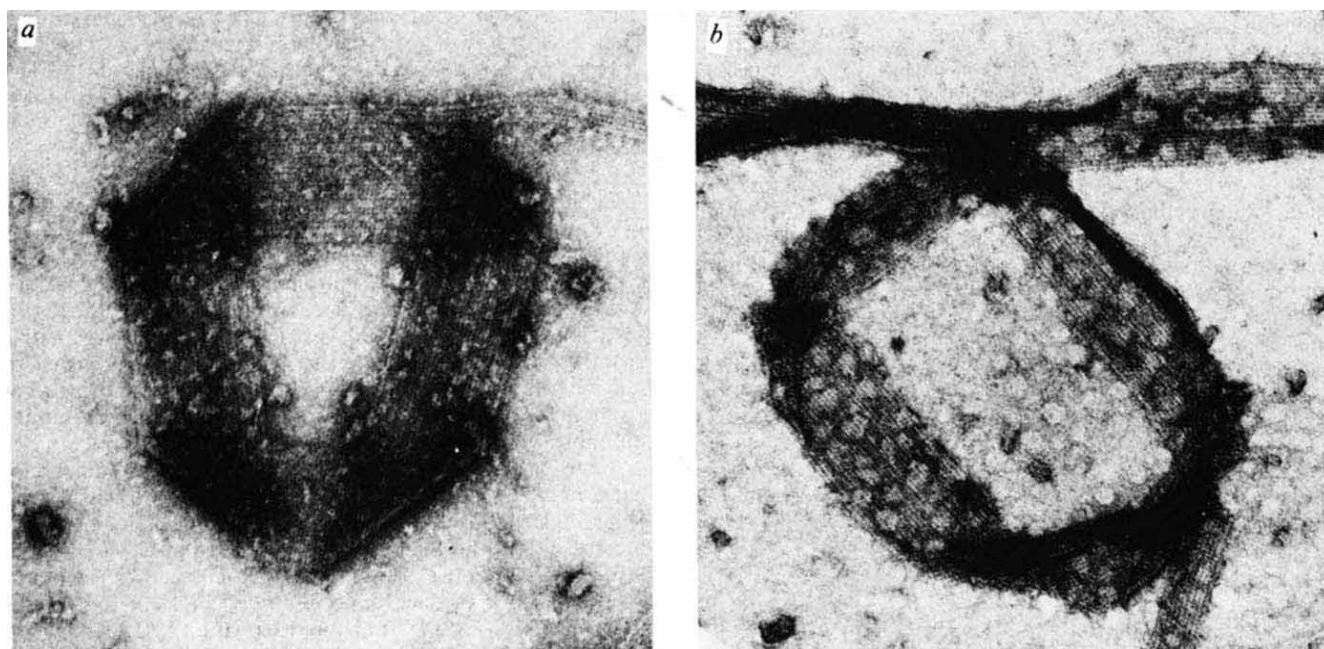


Fig. 2 *a*, Hoop opened up with all sides flattened on the grid. Adjacent stretches are alternating up and down ($\times 140,000$). *b*, Hoop with adjacent stretches facing the same way up. The outer rim is folded over, away from the grid, as established by platinum shadowed specimens. From the inclinations of the (–3, 1) striations it follows that the outside of the hoop, which faces the carbon, corresponds to the inside of the microtubules. This conclusion is supported by the fact that the (–3, 1) striations in the hoop and those in the adjacent sheet which is folding into a tubule, are of opposite inclinations ($\times 140,000$).

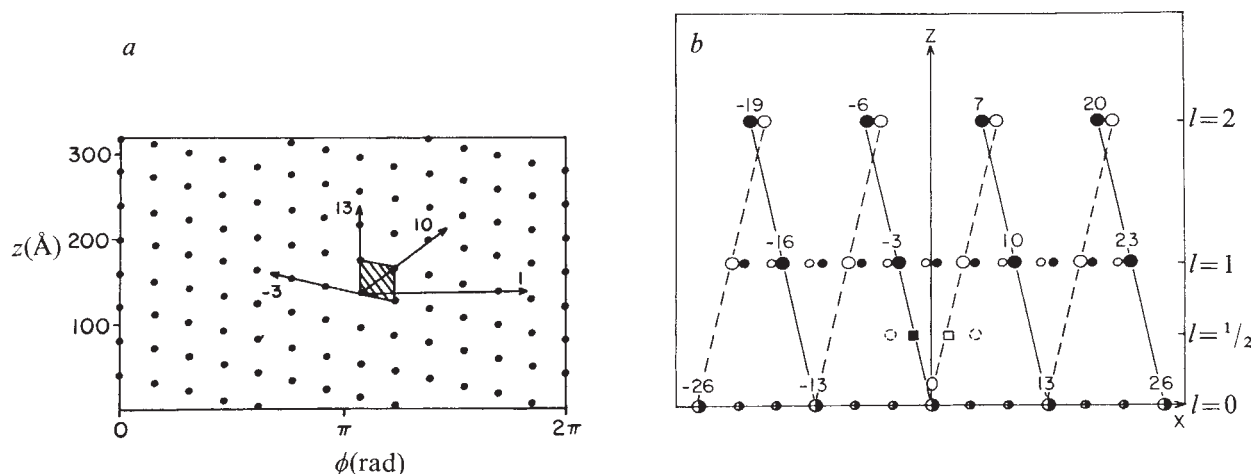


Fig. 3 *a* Microtubule surface lattice according to Amos and Klug⁴ as viewed from outside. Directions of main helical families are indicated. There are 13 vertical protofilaments, staggered by 0.9 nm to produce a left-handed 3-start or a right-handed 10-start helix. The 1-start genetic helix does not connect adjacent subunits, so is of no physical significance. Area of unit cell is shaded. *b*, Diffraction pattern expected for flat (two-dimensional) sheets of protofilaments. Microtubule helix nomenclature is used to characterise the diffraction spots: (n, l) refers to the number of helices in a given family, l is the layer line indicating their vertical separation, c/l ($c = 4$ nm). The equatorial spot $(13, 0)$ comes from the lateral separation of protofilaments; $(26, 0)$ is the second order showing a deepening of the groove and/or a protofilament splitting^{1,6}. The $(-3, 1)$ spot on the first layer line arises from the shallow left-handed 3-start helix which is relatively enhanced in electron images, while the $(10, 1)$ spot corresponds to the steeper 10-start helix dominating the X-ray pattern. Open and filled circles correspond to the sheet facing up or down. The spacings of spots in the two lattices (solid and dotted lines) are the same, but inclinations are symmetric about the meridian (Z -axis). Sheets or hoop top views have either open or filled reflections, hoop side views have both. On the equator ($l = 0$) both lattices are superimposed. The ghost reflections arising from tripling are indicated only on the equator and the first layer line. Numbers refer to the rotational symmetry n of the corresponding structural feature in the microtubule. The quadratic spots on $l = 1/2$ indicate the B-type superlattice found in hoops, the dotted circles show the positions expected for an A-type bonding (not observed). Hoops are wound up from shallow spirals of parent sheets so that in side views the solid and dotted lattices are slightly rotated ($1-2^\circ$) with respect to one another.

patterns show a series of ghost reflections dividing the space between main spots into three parts along the horizontal direction (Fig. 1*b*). Hoops smaller than about 10 protofilaments do not show tripling in a systematic manner, nor do the sheets, although some separation into triplets can occasionally be found. In particular, parent sheets forming hoops usually contain between three and seven protofilaments, not only three or multiples thereof. Tripling thus seems to represent an inherent bonding property of cytoplasmic tubulin.

The physical basis for tripling is not known. Flagellar outer doublet microtubules have a partition wall between A and B tubules consisting of three protofilaments of particular stability⁷. Their coherence is attributed to extra protein components⁸. It is possible that even cytoplasmic tubulin has some propensity to form a 'partition wall'. A second inference comes from duplex tubules⁹; that is, cytoplasmic microtubules coated with an outer layer of protofilaments running along a flat helix. The diffraction pattern shows a 15-nm layer line which could mean that the duplex helix repeats after three turns or that the turns are grouped together in threes.

Another unusual bonding property of the hoops is indicated by a very weak 8-nm layer line (Fig. 1*b*). In their analysis of flagellar microtubules, Amos and Klug⁴ derive two different surface lattices for the positions of $\alpha\beta$ -heterodimers. In both cases the dimers are assumed to lie along the protofilaments ($\alpha\beta\alpha\beta \dots$). Outer doublet A tubules have alternating subunits along the 3-start helices ($\alpha\beta\alpha\beta \dots$). Thus along the 10-start helices the bonding is either ($\alpha\alpha\alpha \dots$) or ($\beta\beta\beta \dots$). For the incomplete B tubules the reverse holds true. These structures are distinguished by the positions of reflections on the 8-nm layer line (Fig. 3*b*). This analysis suggests that the arrangement of dimers in the hoops is similar to that in the flagellar B subfibrils.

Identical bonding of dimers, assuming a 13-protofilament microtubule¹⁰ is possible only with the A-type subunit arrangement. Symmetry arguments therefore suggest that cytoplasmic microtubules have A-type bonding. But, the

B-type packing observed with hoops, if present in microtubules, would imply non-identical bonding of subunits. X-ray patterns from microtubules support this possibility⁶. Thus, both the tripling of protofilaments and the B-type lattice in the hoops suggests a close relation between cytoplasmic and flagellar tubulins.

These studies were undertaken to obtain large, well ordered aggregates of tubulin to aid in the interpretation of X-ray patterns⁶. The hoops contain many unit cells (up to 20,000), and even when negatively stained, their diffraction patterns usually extend to the second and occasionally to the third layer line. This should make them useful in extending the resolution of the existing microtubule model.

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Comparison of bacterial and animal rhodopsins by hydrogen exchange studies

RHODOPSIN is deeply embedded in a hydrocarbon membrane, yet hydrogen exchange (HX) studies show that about 70% of its peptide hydrogens are freely exposed to solvent water¹. This contrasts with other membrane-bound proteins and even aqueous proteins which generally involve about 70% of their peptide hydrogens in internal H-bonding. It has been suggested that the large fraction of disordered polypeptide chain indicated for rhodopsin resides in and acts to stabilise a channel of water penetrating into the membrane, and further that this channel must be quite large, perhaps 12 Å or more in diameter¹. Unwin and Henderson² described an electron diffraction model for the purple membrane protein of *Halobacterium halobium* (bacteriorhodopsin) which compares interestingly with the suggested structure of animal rhodopsin; membrane-embedded bacteriorhodopsin is arranged in a three-molecule circle surrounding an open space 20 Å in diameter. This space, however, is filled not with water but with lipid, and the protein walls of this blind channel are formed of a double rank of α helices which account for most of the bacteriorhodopsin polypeptide chain, so that bacteriorhodopsin, unlike animal rhodopsin, must be as extensively H-bonded as is the usual protein. We report here a HX study of bacteriorhodopsin. In agreement with the electron diffraction model, our HX results show that this protein, unlike animal rhodopsin, has about 75% of its peptides internally H-bonded. These and previous results, however, suggest a fundamental structural similarity between the two rhodopsins.

HX curves for bacteriorhodopsin in purple membrane are shown in Fig. 1. The slowly exchanging hydrogens measured come from internally H-bonded peptide groups of the membrane-bound protein (for discussion see ref. 1). To measure these, they were initially labelled, before the exchange-out experiment started, by incubating membrane suspensions in tritiated water for long periods at high pH to promote fast exchange. Exchange of the slow hydrogens that contribute to Fig. 1 was not characterised in any detail because we are more interested at this stage in the number of freely exposed peptide hydrogens in bacteriorhodopsin in order to compare this with the diffraction model and with animal rhodopsin.

To measure the faster exchanging, freely exposed peptide hydrogens, the earliest part of the curves in Fig. 1 must be studied. It is difficult to measure with good resolution these faster hydrogens above the large background of slow hydrogens. High resolution can be achieved by use of a limited exchange-in manipulation, described before (for example, ref. 3), which suppresses the background of slow hydrogens. Samples at pH 5 and 0 °C, where the free peptide half-time is close to 1 min, were initially exchanged-in for only 5 min, then quickly passed through a Sephadex column to terminate labelling and initiate exchange-out. This limited incubation period labels all the free peptides but only a greatly reduced number of the more slowly exchanging sites. The measured exchange-out data for the partially labelled protein (Fig. 2a) are fitted by a fast phase with the expected free peptide half-time and a slower phase with no predetermined parameters. The isolated fast phase is shown in Fig. 2b. Essentially the same procedure was repeated at pH 4.7 where peptide HX rate is twofold slower (Fig. 3). In both cases the data indicate ~50 hydrogens per molecule exchanging at the expected free peptide rate. One can add 10 prolines to the unbonded residues. These amount to 25% of the total of 242 peptide groups⁴ in the molecule. This value is in line with the small fraction

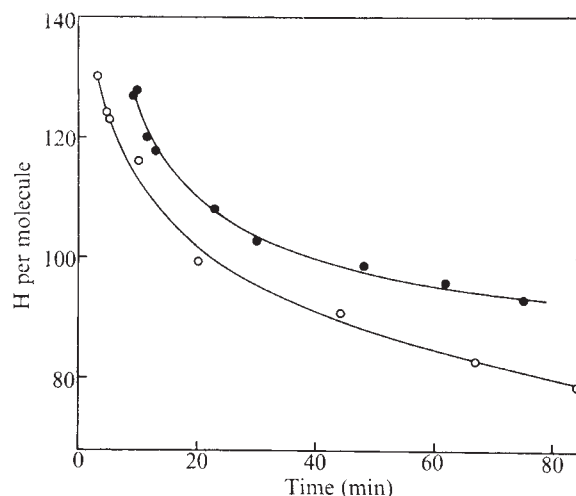


Fig. 1 Hydrogen-tritium exchange-out data for purple membrane suspensions at pH 5.0 (○) and pH 4.7 (●). The bacteria were grown and the membranes were isolated according to Osterholt and Stoekenius¹⁰. After exchange-in at pH 10.5 (2 mM alanine buffer) for 5 d at 4 °C, the purple membrane suspension was adjusted to the appropriate pH and passed through a medium Sephadex-G25 column (at low hydrostatic pressure; 0 °C; pH 5.0, 10 mM cacodylate or pH 4.7, 5 mM citrate) to remove free tritiated water and initiate exchange-out. HX methods were detailed elsewhere¹¹. Two to three drop fractions collected from the final columns were diluted to 0.5 ml with 0.1 M phosphate buffer at pH 7.4, and maintained at 0 °C in order to measure absorbance. (Absorbance measurements decrease slowly with time at room temperature and are slightly dependent upon pH and salt concentration.) Protein concentration in the samples was then computed using a molar extinction coefficient of 7.2×10^4 at 570 nm, determined using the retinaloxime method of Wald and Brown¹² and the thiobarbituric acid method of Futterman¹³. (Total range on 4 preparations was 7.0 – 7.5×10^4 .) Bound tritium in the samples was assayed by liquid scintillation counting. These data were used to compute the number of hydrogens per molecule still unexchanged as a function of exchange-out time.

of free peptide hydrogens generally seen in other proteins and is consistent with the small fraction expected from the Unwin-Henderson model², but it is very different from the value of ~70% free peptides found for animal rhodopsin.

The total number of hydrogens measured in these experiments can be obtained with best accuracy by considering Figs 1 and 2 together. Figure 2a gives the number that exchange in 5 min at pH 5.0 (since initial exchange-in time was 5 min). The number of hydrogens that exchange between 5 min (pH 5) and very much longer times can be obtained from the 5-min point on the pH 5.0 curve of

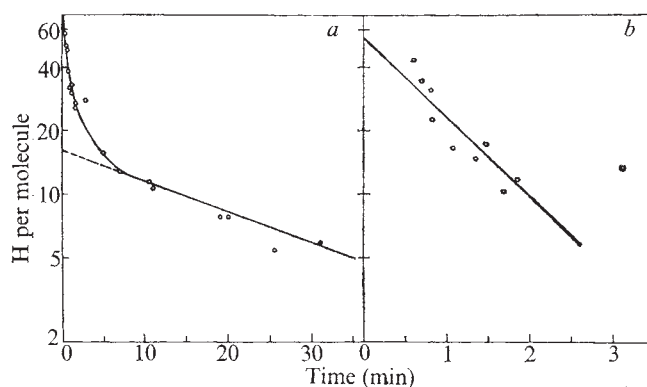


Fig. 2 Limited exchange-in/exchange-out data at pH 5.0 and 0 °C. Purple membrane suspensions were labelled at 0 °C for only 5 min at pH 5 (10 mM cacodylate) and then passed quickly through a Sephadex column to initiate exchange-out. A semilog plot of the resulting data is given in *a* and the isolated free peptide phase, obtained by subtracting the slow phase from the early time data, is in *b*.

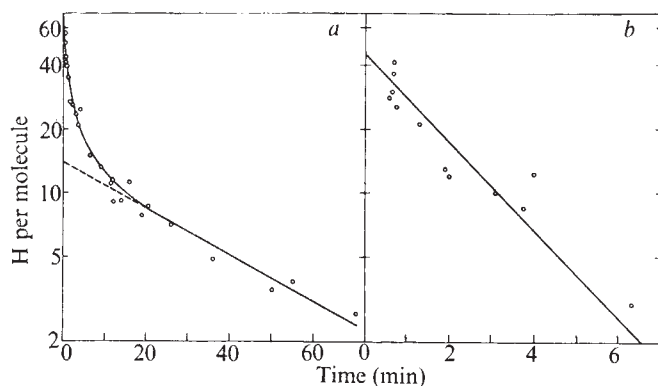


Fig. 3 Limited exchange-out data at pH 4.7 and 0°C. Exchange-in was for 10 min at pH 4.7 (5 mM citrate) and subsequent treatment was as for Fig. 2.

Fig. 1. The sum of the faster and the slower hydrogens then yields the total number. Similarly, an independent estimate can be obtained from the pH 4.7 data (Figs 1 and 3a). The total number found, ~190 per bacteriorhodopsin molecule, is significantly lower even than the total number of amide hydrogens present (232 peptide H plus from 0 to 64 side chain amide H^{1,3}; degree of amidation in unknown). In similar studies with other proteins including membrane-bound animal rhodopsin, the number of hydrogens measured was experimentally identical with the number of amide hydrogens present. The resistant sites in bacteriorhodopsin which could not be labelled by the rigorous exchange-in conditions used here seem to carry the most slowly exchanging peptide hydrogens as yet indicated for any protein, with an exchange rate slower than the expected free peptide rate by a factor of 10¹⁰ or more. In view of the structure and placement of bacteriorhodopsin, this result is perhaps not too surprising.

These results indicate that bacteriorhodopsin has about 60 free peptides and therefore 180 internally H-bonded ones. This compares with the minimum number of 150 peptide H-bonds required by the Unwin-Henderson model² (to generate seven helices of seven turns each with the final turn non-bonded). Previous results¹ show the larger animal rhodopsin molecule to have about 100 H-bonded peptides, and just over 200 free peptides.

Some similarities between bacterial and animal rhodopsins have been pointed out by Osterhelt and Stoeckenius³, including the use of a *cis*-retinaldehyde as a chromophore, the retinal to lysine Schiff-base linkage, and the effect of the protein on the retinal spectrum and on its chemical accessibility. Further, there is evidence that animal rhodopsin, like bacteriorhodopsin, has a quantity of α helix oriented predominantly normal to the membrane surface⁴, and there is the suggestion that animal rhodopsin, like bacteriorhodopsin, may exist as aggregates of several monomers⁵. HX results point to another mild coincidence; though animal rhodopsin is considerably larger than bacteriorhodopsin, the extra length of polypeptide chain in animal rhodopsin has no regular secondary structure so that the structured regions of these two proteins are of roughly comparable size.

Considering possible relatedness of these proteins, it is interesting to examine the bacteriorhodopsin model for clues to the structure and perhaps the function of animal rhodopsin. We note the coincidence involving the previously suggested large channel in animal rhodopsin and the 20-Å 'channel' found in bacteriorhodopsin. While the channel in bacteriorhodopsin is filled in with lipid², it has been suggested that animal rhodopsin maintains a functionally permeable channel by placing many of its extra amino acid residues within the channel with peptide groups

exposed to maintain a polar milieu, therefore an aqueous filling¹. The apparent structural similarities mentioned above suggest a supporting structural framework like that in bacteriorhodopsin for the maintenance of the channel in animal rhodopsin.

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Time-resolved resonance Raman spectroscopy of bacteriorhodopsin

BACTERIORHODOPSIN is found in purple patches on the cell membrane of halophilic bacteria¹. Similar in structure to rhodopsin (but differing in function), it consists of a retinal-protein complex. Bacteriorhodopsin is a photosynthetic system which, when it absorbs light, passes through a cycle of several intermediates (Fig. 1) approximately 200 times per second, pumping protons from the inside of the bacterial cell to the outside. The resultant electrochemical gradient drives the phosphorylation of ADP to ATP (ref. 1). The intermediates have been identified by measuring their relatively broad structureless optical absorption spectra²⁻⁴ as a function of time after a photolytic flash. The most precise structural information has come from resonance Raman spectra⁵⁻⁸ taken with continuous wave (cw) optical excitation. These studies have

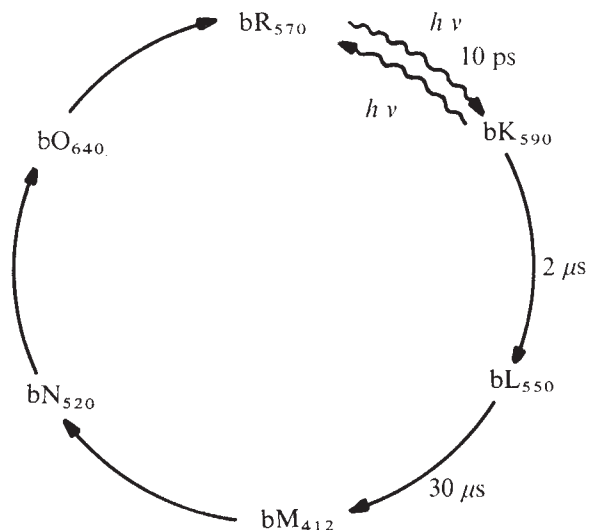


Fig. 1 Photochemical cycle of bacteriorhodopsin (see refs 2-4). Subscripts refer to the optical absorption maxima of each species (nm). Approximate half times are given for room temperature. One cycle is completed in several milliseconds.

provided information about the bR_{570} and bM_{412} intermediates only, since they have appreciable steady state concentrations at room temperature under constant illumination. Resonance Raman spectra of these species have also been obtained at liquid N_2 temperatures and in ether saturated solutions. These conditions, however, may not be representative of actual physiological conditions. We report here a time-resolved resonance Raman spectrum of an intermediate with a risetime of < 6 ns. Our data show a decrease in the $C = C$ stretching frequency by $10\text{--}20\text{ cm}^{-1}$. From the known inverse correlation between the $C = C$ stretching frequency and the position of the absorption maxima⁷ for a series of model retinal Schiff bases in various solvents⁹, the observed $C = C$ Raman band was assigned to the bK_{590} intermediate, which is known to have a risetime of 10 ps^4 .

Time-resolved resonance Raman spectra of bacteriorhodopsin were obtained using a nitrogen pumped tunable dye laser (100-kW 5-ns pulses) at various excitation wavelengths near the maximum of the action spectrum of bacteriorhodopsin¹⁰. The gated detection system involved a PAR optical multi-channel analyser coupled to low (15 cm^{-1}) and moderate (3 cm^{-1}) resolution monochromators using Corning cutoff filters to eliminate scattered radiation at the laser frequency.

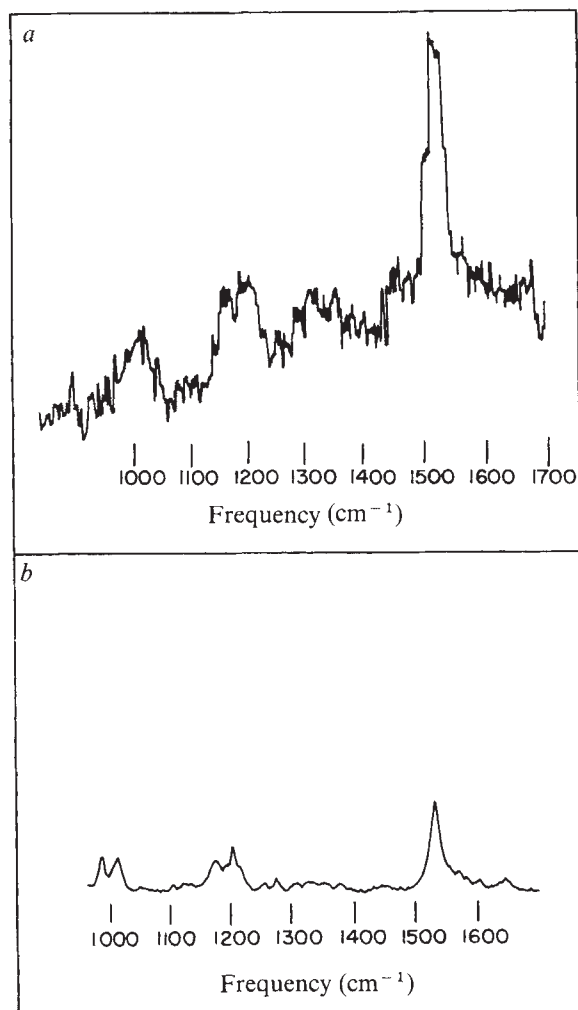


Fig. 2 *a*, Time-resolved (pulse-excited) and *b*, cw resonance Raman spectra of bacteriorhodopsin. The purple membrane fragments were extracted from *Halobacterium halobium* S-9 by the method of Becher and Cassin¹¹ and suspended in 4.3 M NaCl and 0.5 M MgSO_4 at concentrations of $60\text{--}100\text{ }\mu\text{M}$. The time-resolved spectrum was obtained with $5480\text{-}\text{\AA}$ excitation with a peak power of 100 kW (average power 10 mW) from a Moletron pulsed tunable dye laser. The cw spectrum was excited with 200 mW of cw $5145\text{-}\text{\AA}$ excitation from a Spectra Physics Model 165 argon ion laser.

The low resolution spectrum with $5480\text{-}\text{\AA}$ pulsed excitation is shown in Fig. 2*a*. The prominent features are bands at $1,010$, $1,210$ and $1,530\text{ cm}^{-1}$. The spectrum can be compared with a spectrum of bacteriorhodopsin taken with the continuous $5,145\text{-}\text{\AA}$ line of an argon ion laser (Fig. 2*b*). This spectrum agrees in all respects with those previously reported in the literature⁵⁻⁸ for bacteriorhodopsin. Considering the lower resolution of the pulse-excited spectrum, the close resemblance of these two spectra confirms that we observed the resonance Raman spectrum of bacteriorhodopsin with a pulsed ns source.

The $1,529 (\pm 3)\text{ cm}^{-1}$ band ($\nu_C = c$) in the pulse-excited spectrum was asymmetric in the low resolution experiment, so we recorded its band shape under higher resolution. The trace in Fig. 3*a* was obtained at an instrumental resolution of 3 cm^{-1} with the pulsed laser at $5,821\text{ }\text{\AA}$. The asymmetry evident in the low resolution scan is revealed to be a shoulder at $\sim 10\text{ cm}^{-1}$ to lower energy than the main band which occurs at $1,528\text{ cm}^{-1}$. The shoulder does not appear in any spectrum⁵⁻⁸ taken with continuous excitation, regardless of excitation wavelength. The shape of the $1,530\text{-cm}^{-1}$ band measured with the $5,145\text{-}\text{\AA}$ argon line is evident in Fig. 3*b*. Thus the shoulder seems to be due to the use of a pulsed excitation source.

We have ruled out the possibility that the shoulder is a trivial experimental artefact due to the presence of structure in the background (scattered laser light and fluorescence), by separately varying the wavelengths of excitation and position of the peak on the vidicon target. Both position and intensity with respect to the main peak were reproducible in these varying conditions. Comparison of spectra taken before and after prolonged irradiation by the pulsed laser showed no change in the relative position or intensity of the shoulder, eliminating a permanent photoproduct generated by the high intensity pulse as the species responsible for the shoulder.

We interpret this feature as arising from Raman scattering by the first intermediate in the photochemical cycle, bK_{590} . The repetition rate of the experiment (30 Hz) is much slower than the frequency of the photochemical cycle of bacteriorhodopsin (200 Hz) and thus prevents the formation of any appreciable steady-state concentration of the intermediates in the cycle. Optical absorption measurements have shown that the bK_{590} intermediate is generated in approximately 10 ps^4 and has a lifetime of $2\text{ }\mu\text{s}$ (ref. 2) at room temperature. Because the duration of the laser pulse is 5 ns , only bR_{570} and bK_{590} could contribute to the Raman spectrum. In addition, it has been shown¹² using optical absorption spectroscopy that the photoreaction $bR_{570} \rightarrow bK_{590}$ is photoreversible and that a photostationary state can be established during a 40-ns pulse with peak powers above several hundred kW. The relative concentration of bK_{590} and bR_{570} in this stationary state is 0.4 . The intensity of the shoulder in our spectrum is approximately 0.4 that of the main peak at comparable ($\sim 100\text{ kW}$) laser power and on a similar time scale.

The frequency difference between the peak and shoulder is consistent both in sign and magnitude with the correlations between optical absorption band maxima and vibrational frequencies reported for model retinal Schiff bases⁹. A 20-nm shift to the red of the absorption maximum of the chromophore would result in a $10\text{--}15\text{ cm}^{-1}$ decrease in the $C = C$ stretching frequency, which is consistent with our data.

Our interpretation of this new feature is consistent with predictions from previous data on the lifetime and optical absorption spectrum of the bK_{590} species. We feel that we have demonstrated the utility of time-resolved resonance Raman spectroscopy in obtaining structural information about short-lived transients in photobiological cycles. Our experiments were carried out with acceptable, though not excellent signal-to-noise ratios, and we are currently redesigning the detection system for greater resolution and sensitivity. By using two pulsed lasers, one for photolysis and one as a probe beam we should be able to obtain the time-resolved resonance Raman spectra of other intermediates in the cycle. Furthermore,

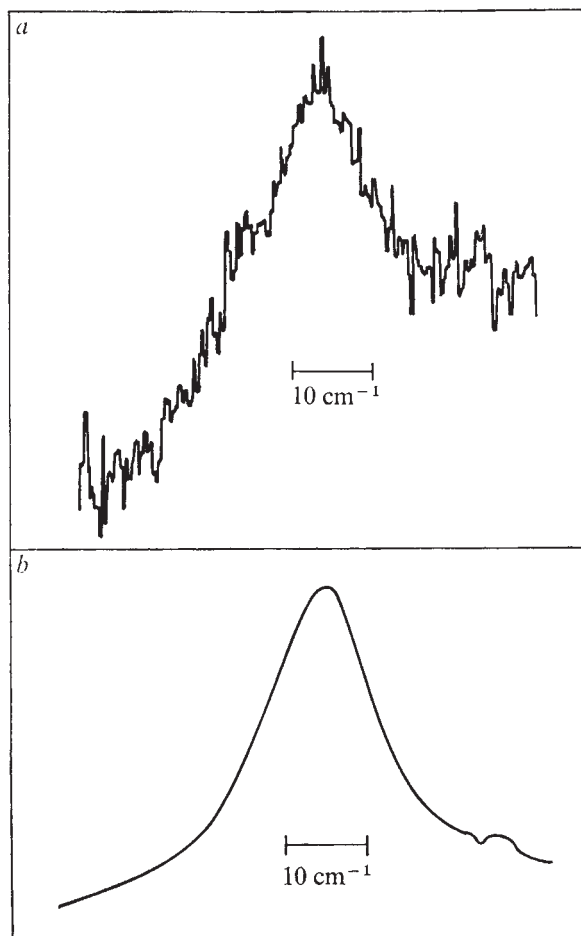


Fig. 3 *a*, Higher resolution of the pulse-excited (time-resolved) resonance Raman spectrum of the $1,530\text{ cm}^{-1}$ band of bacteriorhodopsin excited with 100 kW peak power of $5,821\text{ Å}$ from the pulsed dye laser. *b*, Expanded view of Fig. 2*b* to show the band shape of the $1,530\text{ cm}^{-1}$ peak in the resonance Raman spectrum of bacteriorhodopsin excited with a continuous source at $5,145\text{ Å}$. The resolution is comparable with that in (*a*).

examination of the other bands in the time-resolved spectra should indicate at which point in the cycle deprotonation of the Schiff base occurs.

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Novel N-terminal protein blocking group identified as dimethylproline

THE N-terminal region of *Crithidia oncopelti* cytochrome *c557* could not be characterised by conventional methods because of an apparently blocked amino function¹. To study this feature the N-terminal tryptic peptide was isolated and 220 MHz proton magnetic resonance (PMR) spectroscopy was used to characterise this peptide and its hydrolysis products. We now report the identification of the novel blocking group as dimethylproline.

The tryptic peptide, known to have the structure X-Pro-(CH₃)₃Lys-Ala-Arg (ref. 1) was exposed to thermolysin cleavage to facilitate isolation of X-Pro-(CH₃)₃Lys. The trimethyllysine was then quantitatively removed with carboxypeptidase B. Amino acid analysis of X-Pro-(CH₃)₃Lys-Ala-Arg showed equimolar quantities of the four amino acids and no anomalous peaks. However, if the acid hydrolysate was separated by electrophoresis at pH 2.0 and the paper was sprayed with thymol blue (0.04% in 1:1 butanol-ethanol containing 0.01 M H₂SO₄) (ref. 2), an additional band was located which gave no colour with ninhydrin and had a mobility 0.9 that of proline. The indicator dye method depends on the buffering action of carboxyl groups; yellow (alkali) spots are seen on a red (acid) background.

The hydrolysis and electrophoresis was repeated on a preparative scale (3 μmol peptide), the band was eluted from the electrophoresis paper (Whatman 3MM), and the material was passed down a Sephadex G-10 column (100×1.5 cm) in 5% formic acid in order to remove material washed from the paper after electrophoresis. The PMR spectrum of this material is shown in Fig. 1 and the

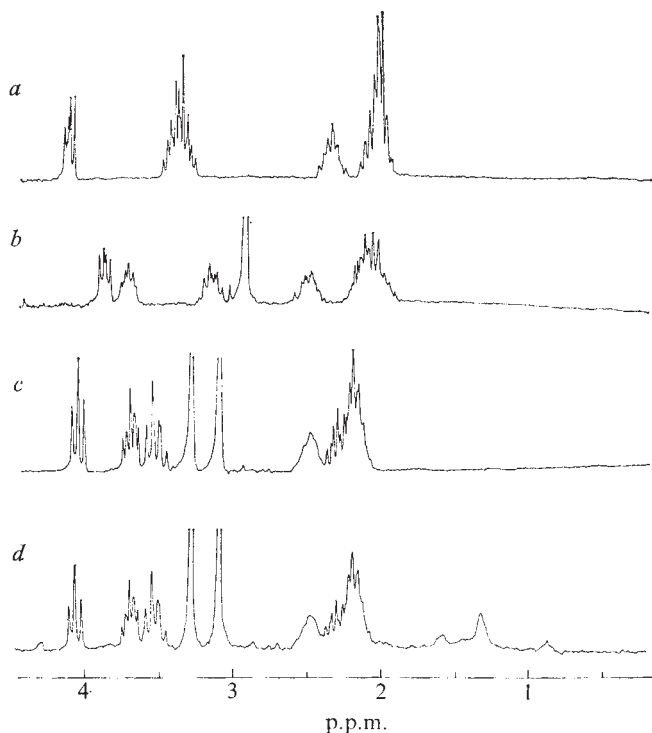


Fig. 1 PMR spectra of: *a*, proline; *b*, monomethylproline; *c*, dimethylproline standard, and *d*, X, isolated from cytochrome *c557*. Solutions were dried in a vacuum from 5% formic acid to remove traces of acetic acid and residues were taken up in 100% D₂O (0.3 ml). Concentrations were approximately 0.5 M for (*a*), (*b*) and (*c*) and 8 mM for (*d*). Spectra were recorded at 22 °C on a Varian HR-220 MHz instrument modified for pulse Fourier transform operations with equipment supplied by Transform Technology Inc., Chemical shifts were measured relative to internal sodium 2,2-dimethyl-2-silapentane sulphonate.

Table 1 Parameters of PMR spectra shown in Fig. 1

Proton Assignment	Proline			Monomethylproline			Dimethylproline			Unknown		
	Chemical shift (Hz)	(p.p.m.)	Area	Chemical shift (Hz)	(p.p.m.)	Area	Chemical shift (Hz)	(p.p.m.)	Area	Chemical shift (Hz)	(p.p.m.)	Area
$\gamma_{1,2} \beta_2$	443	2.01	2.92	456	2.07	2.82	479	2.18	2.9	479	2.18	2.95
β_1	511	2.32	0.99	542	2.46	1.02	543	2.47	0.83	534	2.43	0.98
Methyl-1	—	—	—	637	2.9	3.0	675	3.07	2.95	675	3.07	3.09
Methyl-2	—	—	—	—	—	—	716	3.25	3.0	716	3.25	3.17
δ_2	738	3.35	2.15	692	3.15	1.02	776	3.53	2.1	776	3.53	2.03
δ_1				812	3.69	1.09	809	3.68		809	3.68	
α	898	4.08	0.93	848	3.85	1.02	887	4.03	1.21	887	4.03	1.12

Areas were normalised to the nearest approximation to integral values.

spectral parameters are summarised in Table 1. Although the spectrum is markedly different from that of proline (Fig. 1a), the chemical shifts and areas of the peaks are consistent with a modified proline structure. The two sharp three-proton resonances occur in a region where methyl protons bound to nitrogen are found, suggesting that the material could be *N,N*-dimethylproline.

Dimethylproline was synthesised by alkylation of proline (50 mg) with methyl iodide (5 ml) in methanol (5 ml) under reflux. Alkaline pH was maintained by periodic addition of methanol saturated with NaOH. The synthetic material was isolated by passage down a Sephadex G-10 column (100×1.5 cm) in 20% acetic acid. It had a mobility on pH 2.0 electrophoresis identical to that of the natural material. The PMR spectrum is shown in Fig. 1c and summarised in Table 1, where it is compared with that of monomethylproline synthesised according to Bowman and Stroud³.

It is clear that dimethylproline was present in the acid hydrolysate of the N-terminal peptide. The small amounts of material at 1.3 p.p.m. have been noted with several peptides purified by paper electrophoresis and this material is not completely removed by gel filtration. The evidence is consistent therefore with dimethylproline as the N-terminal blocking group of cytochrome c557 and the N-terminal region having the structure.



There remains the possibility that acid hydrolysis had modified the amino acid of the peptide to give dimethylproline. This possibility could not be investigated by enzymatic digestion as the $(\text{CH}_3)_2\text{Pro-Pro}$ bond was resistant to a wide variety of exo- and endopeptidases. We have therefore examined the problem by comparing the PMR spectra of the natural peptides with that of synthetic dimethylproline. These results, which will be presented elsewhere, are consistent with the presence of dimethylproline in peptide linkage.

Dimethylproline is a novel structure in proteins. It was shown by Vickery⁴ to be present in certain plants as the free amino acid and was named stachydrin. It presents an interesting problem in sequence determination and it is possible that it or other α -N-methylated amino acids may occur in other proteins. Because the amino group is no longer ionisable in these compounds they are invisible or unreactive using conventional N-terminal methods. The indicator dye method² affords a convenient means of visualisation, which was used for the identification of N-terminal amino acids by chemical methylation².

The N-terminal region of cytochrome c557 is ten residues longer than that of the vertebrate cytochromes c. It is possible that this 'tail' and similar 'tails' in plant cytochromes c (which are acetylated)⁵ and fungal cytochromes c (in which the N terminus is free)⁶ form relatively free-moving projections from the N-terminal α -helical region common to all cytochromes c. Perhaps the susceptibility to methylation of this region in cytochrome c557 is due to its

detachment from the globular structure. This may also explain why, during purification, a fraction of cytochrome is always isolated which has lost the five N-terminal acids, presumably by a tryptic-like hydrolysis¹. It is interesting that the lysine residues which are sometimes found methylated in cytochrome c sequences, residues 72 and 87 (ref. 7), are very prominent side chains in the tertiary structure models⁸ and project out from the globular structure more than other lysines. Thus we propose that methylation of lysine, and in this cytochrome N-terminal proline, may be coincidental rather than specific and may depend only on the extent to which the susceptible group is free from the main protein structure.

A striking parallel to the case of cytochrome c577 exists in the myosin alkali light chains that have been sequenced⁹. Frank and Weeds note that the N terminus of the light chain A1 is blocked. This chain is 40 residues longer than the otherwise identical light chain A2; 25% of these 40 residues are proline and the N-terminal region is X-Pro-Lys. The peptide mobilities do not seem consistent with a methylated proline but an N-methylated acidic amino acid does seem a possibility. It would be interesting to re-investigate this region using methods for identifying hydrolysis products with free carboxyl groups.

The proline-rich nature of the N-terminal tail of residues is shared by the plant cytochromes. It is possible that all cytochromes at synthesis contain a proline-rich segment which might be necessary for setting the phase of the N-terminal helix perhaps for folding of the molecule or for the addition of the haem group. This segment may be retained in some and hydrolysed away in others depending on whether blocking of the N terminus had taken place.

Alternatively, a proline-rich segment may be conserved to minimise this very risk that the molecules should be digested by exopeptidases, since proline bonds are notably resistant to many proteases.

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matters arising

Middle Pleistocene stratigraphy in southern East Anglia

ROSE, Allen and Hey¹ proposed a revised chronology for the Middle Pleistocene, based largely on the presence of a rubified *sol* said to be developed in a temperate climate, but no supporting chemical or palynological evidence was produced.

Extensive surveys conducted by ourselves and colleagues (Institute of Geological Sciences, Geological Survey Sheets 223, 240, 241) have shown that the following sequence of drift deposits is present.

The sequence below is the optimum

suggested by Rose, Allen and Hey¹ is questionable for an area near the margin of an active ice-sheet. There is evidence for the former presence of an active ice sheet in the Thames Estuary² area which may have influenced the outwash drainage of the main East Anglian ice and thus account for the deduced north-eastward flow¹ of the lower gravels.

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Interpretation	Lithological unit
Weathered profile or solifluction deposits	7, Heterogeneous brown flinty sandy clay
Late phase fluvioglacial deposits	6, Poorly sorted sands and gravels
Till, dominantly of the lodgement type	5, Chalky boulder clay variably decalcified at base
Flow till	4, Brown and grey mottled sandy clay with angular and rounded flints, involution structures
Proglacial lake deposits	3, Laminated sands, silts and clay
Proximal outwash braidplain deposits with local flow tills	2, Sands and gravels with localised developments of (4) and (5), including the basal chalky Maldon Till which may be of the lodgement type
Distal outwash deposits	1, Fine micaceous sands

development but field evidence shows elements of this succession consistently through the area. All these deposits (1–6) are therefore considered to show integral parts of one (Anglian) glacial cycle of events.

A reddish colouration is developed at a number of horizons particularly where sandy clays have suffered remobilisation or eluviation has taken place from the lowest clay bed in the sequence. The eluviation process was apparently accompanied by dissolution of limestone material from the permeable beds where the ground-water was not lime-saturated. It seems unlikely that each of these horizons could be regarded as indicating a warmer period.

The thin and localised development of the Anglian fluvioglacial deposits

ROSE, ALLEN AND HEY REPLY—In response to Lake *et al.*¹ we wish to clarify the following points:

(1) Recognition of the temperate palaeosol is based not only on red colouration but on clay enrichment, iron enrichment, and clay skins on the upper sides of pebbles. These properties are considered characteristic of the illuvial horizon of a *sol lessivé* formed over a long period in a humid warm temperate climate. We are aware that iron stained horizons are common at many levels in the lower sand and gravel unit, but unlike the palaeosols horizon, these levels are not accompanied by a significant clay enrichment or clay skin development, and their colour does not reach levels of redness as high as 2.5 YR or 10 R. These iron-stained horizons are not

continuous and are not developed at any particular level, but are specifically associated with sedimentary structures along which ground water drainage has been localised. The properties of the rubified *sol lessivé* are developed independently of the sedimentary structures and the original particle size characteristics.

(2) Lake *et al.* state that “sandy clays have suffered remobilisation, or eluviation has taken place from the lower clay bed in the sequence”, to account for the “reddish colouration”. If these processes were responsible, however, reddish colouration should be continuous along the interface of the sands and gravels and the overlying till. This is not the case. At certain localities the palaeosols horizon is cut-out by till. At other localities it is overlain by the upper sand and gravel unit and is thus not associated with a ‘clay bed’. As the palaeosols horizon is cut-out by glacial and glaciifluvial erosion, and as blocks of the palaeosol form erratics in the overlying till, rubification has occurred before ice invaded the locality.

(3) The observation that “the eluvial process was apparently accompanied by dissolution of limestone material from the permeable beds where the ground-water was not saturated” is incorrect, as fresh, hard limestone pebbles have been observed in all parts of the lower sand and gravel unit.

(4) An arctic structure soil is superimposed on the *sol lessivé*. This consists of an horizon of involutions, ice wedge casts, and sand wedges and is associated with coversand, loess, and wind polished stones. This indicates a long period of sub-aerial weathering in addition to the *sol lessivé*, and also demonstrates that the sequence of deposits were not formed in a single “glacial cycle of events”.

(5) The revision of the stratigraphy is not solely based on the recognition of the palaeosols horizon. The glaciifluvial sands and gravels above the palaeosols can be distinguished from the underlying periglacial river deposits on the basis of particle size, stone content, primary sedimentary structures, secondary sedimentary structures, palaeocurrent directions², and heavy mineral assemblage (J. A. Catt, personal communication). The heavy minerals in the lower sand and gravel unit show a restricted residual suite, whereas the range

of heavy minerals in the upper sand and gravel unit is greater and many readily-weatherable minerals are included. This suggests that the heavy minerals in the upper sand and gravel unit have been enriched from a new source, such as would be associated with a glacial event. Also the frequency of volcanic rocks, quartz, and quartzite is much greater in the lower sand and gravel unit than in the till. Thus it is impossible to argue that these materials could have been derived either from the till or from the ice which deposited the till.

(6) We are interested to know on what basis active ice in the Thames would affect outwash drainage as far away as Bury St. Edmunds.

(7) Anglian glacial deposits are restricted because most of the melt-water beneath the ice was concentrated in 'tunnel valleys' related to a low sea level³. The relative scarcity of glacial deposits in an ice-marginal location is not peculiar to southern East Anglia. It is also characteristic of the southern margin of the Devensian ice sheet.

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The cause of Forbush's 20-year wave

LEVY¹ has suggested an explanation of Forbush's² 20-yr wave in the galactic cosmic-ray diurnal anisotropy. Levy states he knows of no other way to account for the variation. This note discusses a previously known alternative mechanism and provides a calculation for the thickness of the heliosheath.

Any mechanism to explain Forbush's 20-yr wave must use the 20-yr oscillatory solar cycle vector dipole field (B_{SD}). To obtain a sinusoidal wave from this vector, it is necessary to have a mechanism which basically takes the

product of B_{SD} with a stationary vector. Levy's mechanism utilises the azimuthal twisting of the interplanetary magnetic field (IMF) lines to obtain an enhanced radial $\nabla \times B$ drift between the positive and negative field of the extended solar dipole. The azimuthal twisting of the field as a result of the solar rotation is important because the perpendicular drift in a radial field would only result in an azimuthal drift of the cosmic rays³ with no outward 20-yr wave. Thus Levy's mechanism takes the product between B_{SD} and the angular velocity vector of the Sun to obtain an effect.

Schatten and Wilcox⁴, however, take the product between B_{SD} and the nearby galactic magnetic field (GMF) vector to obtain their effect. In this model, cosmic rays either gain access to the heliosphere at high (or low) heliographic latitude depending on whether the extended IMF connects to (or is closed from) the GMF. This causes a 20-yr streaming of cosmic rays away from (or toward) the Sun along the IMF of the type Forbush observes, and Duggal and Pomerantz⁵ confirm.

Matters arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

It is interesting that this latter view, if correct, enables a calculation of the thickness of the heliosheath. The cosmic rays producing the Forbush effect have an energy of around 25 GeV (refs 1 and 2). Thus their rigidity is $R V = 300 H \rho$ (H in gauss, ρ in cm).

The heliosheath fields are most effective at filtering cosmic rays with gyro-radii comparable to the heliosheath thickness $\rho \sim d$. An extension of the Archimedean spiral IMF suggests H may be as low as 6×10^{-7} G whereas the galactic magnetic field is nearer 10^{-6} G (ref. 6). Equating ρ to d in the above equation, we find for d , a value of 1.4×10^{14} cm ~ 10 a.u. This is in fact approximately the value suggested for the heliosheath thickness⁷. Undoubtedly Pioneer missions to the outer planets may add to our knowledge about what is happening in this interesting region of space.

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LEVY REPLIES—The suggestion put forward by Schatten and Wilcox¹, that the solar-magnetic-cycle wave in the diurnal variation of energetic cosmic rays results from alternating connection and disconnection of the interplanetary and galactic magnetic fields, is an interesting one that deserves quantitative discussion. Although this is not the place to discuss the idea in detail, one remark does seem in order.

As I understand Schatten's present calculation, particles with cyclotron radii that are small compared with the characteristic length scales in the magnetic-field reconnection region will efficiently follow the field lines and thus will show the largest effect of field line topology. Particles with increasing energy, and thus increasing cyclotron radii, will be less inhibited in their motion by the details of the field-line topology in the heliosheath reconnection region. Thus it seems to be a natural consequence of Schatten's and Wilcox's proposal, in its present form, that the amplitude of the variable anisotropy is a monotonically decreasing function of particle energy. The existing, but sparse, information on this point² suggests that this is not the case. The amplitude of the varying anisotropy seems to increase with energy in the range below 20-30 GeV and to decrease at larger energies. This behaviour is consistent with that predicted as a result of particle drifts³ in a large-scale coherent interplanetary magnetic field⁴.

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reviews

Adaptation to marine life

R. P. Dales

Adaptation to Environment: Essays on the Physiology of Marine Animals. Edited by R. C. Newell, Pp. 539. (Butterworths: London and Boston, Massachusetts, November 1976.) £25.

THIS book comprises nine separate reviews: Adaptations to intertidal life by the Editor himself; Settlement responses in marine organisms by Professor Crisp; Primitive respiratory mechanisms by C. P. Mangum; Physiological adaptation to life in estuaries by A. P. M. Lockwood; Rhythms, behaviour and reproduction in marine animals by Professor Naylor; Vision in pelagic animals by J. K. Bowmaker; and three chapters by P. W. Hochachka and G. N. Somero: Biochemical adaptations to pressure; Enzyme and metabolic adaptations to low oxygen, and Biochemical adaptations to temperature.

Nine chapters within the compass of over 500 pages has provided each author with sufficient space to review in some detail the current concepts and knowledge in their own field. Any of these reviews might have appeared in either of the volumes of reviews in marine biology currently being published by Academic Press or Allen and Unwin. Such a volume as this may be justified by the amount of work being produced or by the individual contributions making a coherent whole.

It is six years since Newell's book on the *Biology of Intertidal Animals* appeared and so much interesting research has been done since then that in many ways I regret that the field was not restricted to environmental adaptations of intertidal animals. This is not to welcome Bowmaker's contribution on vision for, although this chapter is perhaps the most isolated in subject matter from the others, a review of this subject was much needed. And, although the literature is less extensive than that reviewed elsewhere in the book, the most significant recent papers are not readily available. It is also a more difficult subject for most students, so that it is good to have this literature authoritatively assessed.

This is an invaluable book to all those interested in the adaptation of organisms to their environment. Much of the contents are familiar, but the

incorporation of recent work and subsequent discussion is valuable for students and teachers trying to keep up-to-date in this field.

Thus Crisp's review of 1974 is amplified, and Naylor's review of rhythmic behaviour is to be welcomed because it brings together various aspects of the subject such as circadian rhythms, vertical migration and reproductive cycles. Mangum's review also pulls together the many recent contributions in the field of respiratory pigment function. This last is particularly interesting for the comparison of intra- and extracellular respiratory proteins and for her ideas on the evolutionary significance of red cells in invertebrates. Lockwood's chapter on the physiology of estuarine organisms is useful too, not only because it is his own account but because other recent reviews and treatises have been more ecological than physiological in approach.

The three chapters by Hochachka and Somero also bring up-to-date the subjects discussed in their recent book on *Strategies* (Saunders: Philadelphia, 1973), in *Comp. Biochem. Physiol.* and in the Society for Experimental Biology Symposium on *Pressure*, edited by Sleigh and Macdonald. Most of the chapter on low O₂ adaptations will be familiar to those who know *Strategies* but the more recent work done both by Hochachka's own group and by de Zwaan's group in Utrecht on bivalves, is included here.

A most useful series of essays on physiological and biochemical adaptation, well printed and illustrated, a book no zoological library can afford to be without. It is a pity that rising costs puts such a book beyond the reach of most individuals. □

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Ion implantation

Ion Implantation of Semiconductors. By G. Carter and W. A. Grant. Pp. viii+214. (Arnold: London, 1976.) Boards £9.50; paper £5.95.

ION IMPLANTATION was for a number of years a specialist subject studied only by research scientists and engineers. The past five years has seen the increased acceptance of the technique in the semiconductor device manufacturing industry and a growing awareness of its potential in other areas. It is not surprising, therefore, that a number of educational establishments have introduced courses on the subject. It is clearly intended that this book should provide a student text for such courses. For this purpose the book is well suited.

It guides the reader in a logical manner through the physics of ion implantation. Starting with very elementary concepts, all aspects of the subject are explained. There is an unfortunate over simplification of the theory of ion range calculations. It has been assumed, without apparent justification, that implanted profiles are symmetrical, whereas the physics of observed asymmetric profiles is well understood.

The greatest shortcoming of the book is in the treatment of the electrical effects of ion implantation in semiconductors. Most of the book could be applied equally well to metals and insulators. As it is the electrical effects in semiconductors that have made them of special technological interest it was very disappointing to find that the discussion on this aspect did not merit a chapter on its own but was included in a rather abbreviated devices chapter. This has meant that important topics such as electrical measurements during annealing have been curtailed and the electrical properties of compound semiconductors virtually omitted. It has also meant that some important device application areas such as threshold changing in MOS transistors were not included.

In summary, the book is likely to be useful as a general introduction to the subject but should not be regarded as being specific to semiconductor applications.

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Chemical carcinogens

Chemical Carcinogens. (ACS Monograph 173.) By C. E. Searle. Pp. xxvii + 788. (American Chemical Society: Washington, DC, 1976.) \$67.50.

THAT most cancer in man is caused by chemicals is now accepted, although the first demonstration of cancer produced by a pure chemical compound was only published in 1930 as a postscript to a paper by E. L. Kennaway. Before that there was evidence that soot, coal tar, pitch, and some oils caused cancer in man; and soon after Kennaway's successful experiment the widely distributed carcinogen, benzo-pyrene, was isolated from coal tar and synthesised. These experiments opened the gates to a flood of research in chemical carcinogenesis. Most cancer in man is caused by chemical substances in the environment and a sure way of preventing cancer is to identify these carcinogens and remove them. Such removal is not always feasible in practice. The most ubiquitous cause of cancer in Britain is tobacco but it seems impossible to prevent smoking. The active chemical carcinogen or carcinogens in tobacco smoke are still not known and attempts to make 'safer' cigarettes are hampered by the lack of knowledge.

The existing knowledge of chemical carcinogens and the research in the problems are so vast that it is difficult for individuals or even teams of workers to cover the subject. The volume under review is an admirable and much needed attempt to present the available facts and some of the theories. The main gap in the book is that inorganic carcinogens, except asbestos, are not considered.

The editor points out that this is not the first effort to list a catalogue of chemical carcinogens. The US Department of Health, Education and Welfare has published a series entitled *Survey of Compounds Which Have Been Tested for Carcinogenic Activity*, some of which were edited by Hartwell and Shubik. The first of the series appeared in 1951 and the last, which is generally available, includes data published in 1971. The International Agency for Research on Cancer in Lyon has published twelve monographs on the *Evaluation of the Carcinogenic Risk to Man*. These monographs are invaluable to research workers and administrators concerned with cancer problems. In very few cases, however, is it possible to evaluate the potential carcinogenic hazard to man of pure chemical compounds.

In addition to the preface written by the editor, which is an excellent review of the present position of the subject, there are 16 chapters written

by 23 experts in their fields. There is less repetition than would be expected with a book written by so many authors. The chapters on "Soots, tars and oils" by Kipling, on "Laboratory hazards" by George and Searle and on "The Bracken Carcinogen" by I. A. Evans and "Asbestos" by Wagner, are each about 10 pages. The chapter on "N-Nitroso Compounds" by Magee, Montesano and Preussmann is an authoritative review of the subject covering 125 pages. In the case of this and other articles it would be of value and interest to know when they were written.

There are also comprehensive reviews

on endocrine aspects, alkylating agents, polynuclear aromatic carcinogens, environmental respiratory carcinogenesis, aromatic amines, carcinogens in plants and microorganisms, and metabolism of chemical carcinogens.

The book is well produced, easy to handle considering its size, and provides an excellent overview and reference work, although it is already out of date in some minor matters.

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Perception handbook

Handbook of Perception. Vol 7: Language and Speech. Edited by E. C. Carterette and M. P. Friedman. Pp. xviii + 501. (Academic: New York and London, 1976.) £16.25.

A SUCCESSFUL handbook has the opposite fate to a 'classic': it is consulted, but not quoted. It is used to settle disputes, to aid memory, and to fill in inevitable gaps in knowledge. To these ends it must be well organised, comprehensive, and authoritative. In psychology, the standard was set by S. S. Stevens's *Handbook of Experimental Psychology* which was first published in 1951. Carterette and Friedman are engaged in the publication of a more ambitious work, a ten-volume handbook intended "for all in the arts or sciences . . . who are interested in human perception". The first six volumes are concerned with fundamentals, and the present volume is the first to be devoted to a wider consideration of the contents of perception rather than particular processes or modalities.

There are fourteen chapters arranged in four parts: fundamental aspects of language and speech; coding, perception, and hearing; disorders of language and speech; and trends in psychological tests of linguistic theory. These headings are not auspicious and certainly do not reflect a natural organisation of the field. The assignment of topics to them turns out to be fairly arbitrary, and the problem of finding one's way through the book is further aggravated by a considerable degree of overlap between the contents of the chapters. Accounts of the production of speech crop up in three different places, varying considerably in detail, and there are numerous cases of brief descriptions of the same work occurring in more than one chapter—for example, the subjective location of clicks, Katz's theory of semantics, and

Winograd's program for understanding natural language.

The book ranges reasonably comprehensively over the major topics in the area, and includes useful chapters on speech perception (C. Darwin), the syntactic analysis of sentences (J. M. Carroll and T. G. Bever), the research methods of psycholinguistics (G. Olson and H. Clark), non-verbal communication (J. Laver), and aphasia (H. Goodglass and N. Geschwind). Somewhat surprisingly, it also has chapters on language teaching and language learning, disorders in speech production, and the general development of language in children. Yet, a number of topics very relevant to perception do not have chapters devoted to them—for example, intonation and tone of voice, the psychology of reading, and computer programs for understanding speech. At a more technical level, the reader will look in vain for an account of linear prediction theory, augmented transition networks, the perception of illocutionary force, and procedural semantics. It is probably no coincidence that each of these topics reflects an increasing concern with psychological processes in real time rather than with the static structures of linguistic theory. Ironically, the chapter that best captures this growing preoccupation, MacNeilage and Ladefoged's excellent discussion of speech production, is concerned with perception in only a tangential way.

In summary, the compilers have solicited a number of authoritative articles from acknowledged experts, but they have failed to cover the field comprehensively and to edit the materials to the best advantage. They have produced a superior symposium rather than an indispensable handbook.

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Problems of memory representation

Processes of Animal Memory. Edited by D. L. Medin, W. A. Roberts and R. T. Davies. Pp. xi+267. (Hillsdale, Lawrence Erlbaum Associates: New Jersey, 1976. Distributed by the Halsted Press Division of Wiley: New York and London, October 1976.) \$19.00; £11.50.

THIS book is useful because it collates a set of papers by authors who have all worked closely together in the past on problems of memory representation. It demonstrates that the problems of inference of the existence and nature of mental representations in animals are, indeed, formidable, but that with ingenuity much can be said about such possibilities, and that perhaps we can no longer talk sensibly about learning theory if we omit them. It also shows that the questions raised when proper investigations are made are far more interesting than those with which psychologists have fobbed off laymen and bored each other for so long.

The first paper by Ruggiero and Flagg directly takes up these issues. It reviews neglected early work, and describes interesting new work by Medin and Davis, among others, which shows that monkeys find some patterns easier to learn than others. This fact seems trite until the data are considered in terms of the regularities of patterns which are particularly striking to animals. It is not simply that such regularities may reflect the organisation of perceptual analyser systems as postulated by Sutherland, Mackintosh, *et al.* The regularities may also usefully be described in terms of the principles of encoding economy which have been so useful in extending the application of information theory to human pattern recognition. We may talk, though still loosely, of an animal making "the best sense" of a pattern, by selectively remembering its critical rather than incidental characteristics.

The application of paradigms and models conventional in the study of human short-term memory to animal work is a major theme of this symposium. A paper by Davis and Fitts shows that the modifications of paradigms required is not as great as had previously been thought. Although the empirical data they present are as yet too scanty for theoretical treatment, they have already shown that provocative similarities and differences in obtained data do not demand separate theoretical constructs for humans and monkeys.

A charming paper by Bolles revives the study of 'insight' in animal learning which has always been a standing chal-

lenge to behaviourists, and which is the current preoccupation of modern systematic theorists such as Levine. There is, perhaps, too much emphasis on the "Garcia effect", or "stomach memory", that is, the tendency of even lower mammals, such as rats, to avoid specific foods when they have been poisoned (non-fatally!) up to an hour after eating them. Demonstrations that rats will avoid eating in one place rather than another under similar circumstances are interesting. But a further demonstration by Riley that animals poisoned after drinking water show subsequent aversion to novel solutions such as saccharine (control data are not quoted) are less interesting than the author supposes. Rats always tend to avoid novel foodstuffs, and show a cautious empirical approach, eating very little until no uncomfortable consequences have ensued on several occasions. Although rat-catchers may feel their darkest suspicions of rodent cunning have been confirmed, it is still too early to take these as

demonstrations of the understanding of 'causal relationships' by lower animals, as Bolles would wish us to do. He is nevertheless quite right to point out that it is time to seriously consider whether the rigid tenets of stimulus-response learning do not require further testing in this regard. The perception of "rules" and "regularities", and the perception of "causality" shade into each other in ways uncomfortable for older theorists.

A concluding paper by Whitlow attempts a direct reinterpretation of the most elementary S-R learning theory, classical conditioning theory, in terms of temporal organisations of events ("episodic memory theory") now current in the literature on human memory. The conceptual failures involved are illuminating. Perhaps as much for the human as for the animal work.

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Whither goest palaeoecology?

Structure and Classification of Paleo-communities. Edited by R. W. Scott and R. R. West. (Wiley: New York and London, 1976.) £18; \$30.

THIS child is the offspring of 15 authors and was born two years after an American Paleontological Society Symposium held at Miami Beach in 1974. The creative impulse was triggered by the question "Whither goest palaeoecology?". The book consists of 11 chapters of which the first four are basically reviews of community concepts in palaeoecology (Kauffman and Scott), two chapters on classification in terms of trophic levels (Scott) or environments (Parker), and a comparison of living and dead assemblages (Macdonald). The remaining seven chapters are more specific studies related only by having a palaeoecological bias. They range from living communities of the S. Californian shelf (Stanton), through Mexican and Californian living and recently dead molluscs and foraminifera (Warme, A. A. Ekdale, S. F. Ekdale and C. H. Peterson), Pleistocene ostracodes from Utah (Lister), Eocene non-marine molluscs from Wyoming and Colorado (Hanley), seven lingulid communities of different ages (West), Pennsylvanian ostracodes from Kansas (Brondos and Kaesler), to Mississippian communities from Georgia (Broadhead).

Understandably definitions of communities differ. "A paleocommunity is the suite of preservable taxa comprising

a community (pvi)"; as for communities they can be regarded as "a unique congregation of diverse organisms having a unique structure based on organism interactions . . ." (p18); "recurrent groups of numerically co-occurring species" (p143); and (p88) "a group of species which are often found living together".

Most of the authors avoided answering the original question of where the subject was going and preferred to explain where they and others had been. And since vertebrates, plants, trace fossils and substantial parts of the invertebrate palaeoecological record were, in effect, not touched on (and in fairness to the authors nor could they have been within the compass of a slim volume) it is not surprising that the product is a ragged patchwork of individual interests. The frequently overlapping references and interests are predominantly American. A *much* more interesting project would have been to pose the same question, quite separately, to another group of 15 palaeoecologists in the States, and comparable groups in say, Canada, Britain, France, Germany and Russia, and then compare the products.

The blurb claims that the book serves as a fascinating historical overview of the significant advances in community ecology and palaeoecology from the 1950s to the present day, and is an unprecedented summary of current knowledge. The publishers have prudently set the price at £18.00.

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Anti-bacterial therapy

The Battle Against Bacteria: A Fresh Look. By P. Baldry. Pp. 179. (Cambridge University: Cambridge, London, New York and Melbourne, 1976). £4.50.

ONLY six of the 15 groups of anti-bacterial drugs now in wide use were available 20 years ago. Since then, however, the spectre of transferable drug resistance has become the constant adversary of microbiologist and clinician alike. These dramatic changes are emphasised by even a superficial comparison of the two editions of Dr Peter Baldry's history of anti-bacterial therapy. The first edition, although published in 1965, took the story only up to the late 1950s and occupied a compact 100 pages. Inevitably its successor is over 70% longer. It describes the evolution of a field which is a fascinating meeting ground of 'microbiology, clinical medicine, biochemistry, pharmacology and molecular biology.

The book is least successful when summarising the history of bacteriology as distinct from therapy. Thus Leeuwenhoek did not see the 'animalcules' which cause disease; he saw only saprophytes and normal commensals. His illustrious predecessor Robert Hooke is not mentioned, even though an engraving from Hooke's *Micrographia* is included (without attribution). Joseph Lister's pioneering bacteriological work—which included isolating the first pure bacterial cultures—also goes unnoticed, as does his even

more relevant observation of 1871 on the antibiotic action of *Penicillium* cultures. Moreover, Lister was not Professor of Surgery at Edinburgh but in Glasgow at the time of his original discovery of antiseptics. The romantic legend of the martyrdom of Semmelweis also needs correction. This tragic pioneer of hand disinfection in obstetrics in fact suffered from chronic paranoia. After increasing insanity necessitated his committal to a mental hospital his death from presumed streptococcal septicaemia—following a hand injury—was supreme irony. For this was the infection he had prevented in thousands of women during childbirth.

The author is on much firmer ground with his main theme—the discoveries of anti-bacterial drugs. There are only two major disappointments here—apart from the lack of any references: the story of the British work on sulphonamides (culminating in M&B 693) is omitted, and the central chapter on Fleming's original observations of 1928 remains unchanged from the first edition. There is no mention of the fascinating revelations published in 1970 by Fleming's colleague, Ronald Hare. In his book *The Birth of Penicillin*, Hare showed that almost every detail of the familiar story is erroneous. Nevertheless, Dr Baldry's book is a good introduction to an enthralling subject. It should attract a wide audience.

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Noradrenaline and adrenergic nerves

Peripheral Sympathetic Neurotransmission. By W. De. Potter. Pp. xv+161. (Arscia Uitgaven, NV: Brussels, 1976.)

THIS book is a strongly personal account of the longstanding research interests of the author and his laboratory. Since he is an acknowledged leader in his field, it is predictably a good account of the biochemical aspects of the synthesis, storage and release of noradrenaline from adrenergic nerves. Factors affecting the storage and release, such as electrical stimulation, and α - and β -receptor-blocking agents, are also described. The relationship between the different noradrenaline-containing particles is discussed. There is a clear and informative diagram at the end of the book which summarises the author's views of the origin and fate of nor-

adrenaline storage particles. The sections on methods are especially good and should be most useful to those engaged in this area of research. The bibliography is fairly extensive (270 references) although it stops at 1973.

My one reservation is related to the title. In my opinion, this title implies a general coverage of all aspects of transmission including information on neuroeffector geometry, the electrophysiology of transmission, and effector cells including 'receptor' characteristics. These topics are not discussed. There is also little account of transmitter inactivation mechanisms. I have, however, no hesitation in recommending this book for its lucid description of storage and release of noradrenaline from sympathetic nerves. It would make a useful addition to the library of anyone working in the field of neurotransmission.

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Electric fields and isolated atoms

Atoms and Molecules in Electric Fields. By N. Ryde. Pp. 455. (Almqvist and Wiksell International: Stockholm, 1976.)

THE effect of external electric fields on the spectra of isolated atoms and simple molecules has been investigated since early this century. In particular the splitting and shifts in spectral lines observed as the external field is increased, resulting from the perturbation of otherwise spatially degenerate levels, have been important in the development of quantum theory. These 'Stark effect' measurements continue to provide important information on oscillator strengths and the assignment of spectra. Increasingly though, interest has shifted to the application of these effects, for example, in astrophysics or plasma studies where the broadening or line shift can be related to the electron density and temperature. There is thus a need, identified by the author, for a critical compilation of the large amount of data amassed in the past forty years for use in these new areas.

The most substantial and useful section of this book forming about 50% of the whole is just such a compilation of the experimental or calculated electric field properties of some fifty atoms and atomic ions, much of this work dating from the 1920–1930s. Spectral assignments are discussed and plots of line displacement with field strength are recorded here. A large part of the remainder of the book is concerned with the quantum theory of these processes. This section begins with a detailed account of elementary perturbation theory and then reviews the application and development of these techniques in a wide range of atomic and molecular situations.

The other chapters in the book dealing with experimental matters and molecular species are rather limited in scope and will be of only limited interest to the type of reader for whom this book is intended. The use of the phrase 'canal ways' throughout the book makes a curious impression. There is no index.

This book will be a useful reference for those using the atomic 'Stark effect'.

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Recent scientific and technical books

Mathematics

- BERGER, J., BUIHLER, W., REPGES, R., and TAUU, P. (edited by). *Mathematical Models in Medicine: Workshop, Mainz, March 1976*. (Lecture Notes in Biomathematics, Vol. 11.) Pp.xii + 281. ISBN-3-540-07802-9. (Berlin and New York: Springer-Verlag, 1976.) DM28; \$11.50.
- BLAKE, Ian F., and MULLIN, Ronald C. *An Introduction to Algebraic and Combinatorial Coding Theory*. Pp.x + 229. ISBN-0-12-103560-3. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1976.) \$9.50; £6.75.
- CARROLL, R. W., and SHOWALTER, R. E. *Singular and Degenerate Cauchy Problems*. (Mathematics in Science and Engineering: A Series of Monographs and Textbooks, Vol. 127.) Pp.viii + 333. ISBN-0-12-161450-6. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1976.) \$14.50; £10.30.
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- de FIGUEIREDO, Djairo Guedes (edited by). *Functional Analysis*. (Proceedings of the Brazilian Mathematical Society Symposium, Campinas, Brazil.) (Lecture Notes in Pure and Applied Mathematics, Vol. 18.) Pp.viii + 325. ISBN-0-8247-6334-3. (New York and Basel: Marcel Dekker, Inc., 1976.) SFRs.82.
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